Isolation and characterization of rice lesion mimic mutants from a T-DNA tagged population*

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Abstract A rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) T-DNA tagged population consisting of about 7000 individual lines was generated and screened for rice lesion mimic mutants in the T_1 generation. Ten lines were found to develop spontaneous lesions in the absence of pathogen infection and displayed distinct lesion phenotypes. These mutants were tentatively designated as *lm1*—*lm10* (for lesion mimic), respectively. Lesion formation of *lm* mutants was developmentally regulated, and all the mutants showed stunted growth and reduced fertility. Genetic analysis demonstrated that all the mutations were recessive, and five partially fertile mutants (*lm4*—*lm8*) were derived from different loci. Mimic lesions occurring on the leaves of *lm* mutants resulted from cell death as revealed by trypan blue staining. Six of them (*lm3*—*lm8*) exhibited enhanced resistance to five bacterial blight isolates, indicating their wide-spectrum resistance to this pathogen. These results imply that some lesion mimic mutations of rice might be involved in disease resistance signaling pathways, and that isolation of these mutated genes may be useful for elucidating molecular mechanisms of plant disease resistance. Among the mutants, only one mutant, *lm6*, was preliminarily shown to cosegregate with the inserted T-DNA in its T_1 generation, making it feasible to isolate the gene responsible for the phenotype of this mutant.

Keywords: T-DNA tagging, lesion mimic, hypersensitive response, *Xanthomonas oryzae* pv. *Oryzae*, *Oryza sativa*.

Programmed cell death (PCD) is a basic biological process that functions in many aspects of plant development. As one type of PCD, hypersensitive response (HR) leads to rapid localized death of plant cells at the infection site by pathogens. A correlation between HR and plant disease resistance has been observed in many plant species. Considerable efforts have been made to dissect signaling pathways and elucidate the role of cell death in plant disease resistance. One way to investigate molecular mechanisms of cell death and disease resistance is to use mutants with a visible lesion mimic phenotype. A large number of lesion mimic mutants have widely been isolated in many plant species, including maize[1,2], *Arabidopsis*[3,4], barley[5], and rice[6—8]. These mutants spontaneously develop HR-like lesions, and most of them exhibit increased resistance to pathogens when lesions are visible or before lesions are formed. Therefore, defense responses normally activated upon infection by avirulent pathogens are constitutively triggered in most of these mutants in the absence of pathogens.

Eight lesion mimic genes have been isolated so far. Among them, five are directly associated with disease resistance and confer improved resistance to pathogens when lesions appear. These are *Arabidopsis LSD1*[9] and *ACD2*[10], maize *Lls1*[1] and *RP1*[11], and barley *Mlo*[12]. However, maize *Les22*[13], *Arabidopsis MOD1*[14], and rice *Spl7*[15] are three lesion mimic genes that are not involved in disease resistance signaling pathways. Their disruptions resulted in the interruption of vital metabolic processes.

T-DNA insertional mutagenesis provides an efficient way to generate loss-of-function mutants and subsequently to isolate corresponding mutated genes. We have previously reported the generation and flanking sequence analysis of a rice T-DNA tagged population[16]. To further unravel the role of cell death in disease resistance to rice pathogens, ten rice lesion mimic mutants from our tagged population were further identified. These mutants exhibited cytological characteristics of cell death. More importantly, six mutants confer high levels of resistance to multiple bacterial blight isolates.

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1 Materials and methods

1.1 Rice transformation, mutant isolation and genetic analysis

Rice transformation was performed as previously described[16]. Transgenic lines in the T_0 and T_1 generations were grown in a greenhouse maintained at 30 ℃ during the day and 20 ℃ at night. The light/dark cycle in the greenhouse was 14/10 h. Lesion mimic mutants were screened in two to five weeks after sowing. The progeny of heterogeneous T_1 plants were used to confirm the lesion mimic phenotypes of ten *lm* mutants under the same conditions as described above.

Partially fertile lesion mimic mutants were crossed with each other. Then, 14-week-old plants of F_1 and F_2 progeny were examined to determine whether they developed lesion mimics.

1.2 Trypan blue staining and microscopy

Trypan blue staining was performed as previously described with slight modification[7, 14]. Samples were covered with an alcoholic lactophenol trypan blue solution (40 mL ethanol, 10 mL water-saturated phenol, 10 mL H_2O, 10 mL glycerol, 10 mL lactic acid, and 0.2 g trypan blue), placed in a boiling water bath for 10 min, and then slow-release vacuum infiltrated for 10 min. Samples were left to stain in the above solution overnight at room temperature. Subsequently, samples were transferred into a chloral hydrate solution (2.5 g in 1 mL of H_2O), heated in a boiling water bath for 10 min, and then left to destain overnight at room temperature. After multiple exchanges of chloral hydrate solution to reduce the background, samples were equilibrated overnight with 70% glycerol and observed with a stereomicroscope (model SZX-12; Olympus).

1.3 PCR primers and reaction

Primers of HygF (5′-TGCGCCCAAGCTGCATCAT-3′) and HygR (5′-TGAACTCACCGCGACGTCTGT-3′) were designed to amplify a coding region fragment of the hygromycin phosphotransferase (*hpt*) gene. The reaction mix (20 μL) for PCR consists of 5—10 ng of rice genomic DNA, 0.2 mmol/L each of dNTPs, 0.2 μmol/L of each PCR primer, 1× PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$) and 0.8 unit of *Taq* DNA polymerase (TAKARA, Dalian, China). Thermal cycling was performed at 94 ℃ for 5 min followed by 35 cycles of 94 ℃ for 45 s, 60 ℃ for 45 s, 72 ℃ for 90 s. After the final cycle, the reactions were maintained at 72 ℃ for 5 min before completion. Reactions were conducted using a PTC-100™ programmable thermal controller (MJ Research, USA).

1.4 Isolation of genomic DNA and Southern hybridization

Genomic DNA was extracted from leaf tissues (1—2 g per plant) as described by Komari et al.[17]. Genomic DNA was digested with *Hin*d III, separated on a 1% agarose gel in 1× TAE buffer by electrophoresis, blotted onto Hybond-N^+ membranes, and hybridized with the [^{32}P] dCTP-labeled *hpt* probe. The *hpt* gene probe was amplified from pCAMBIA1301 (http://www.cambia.org.au/) with primers HygF and HygR, and labeled with Primer-a-gene Labeling System (Promega, Madison, USA). All Southern analysis procedures were carried out as described by Kang et al.[18].

1.5 Rice bacterial blight inoculation

Inoculation experiments were performed according to Yin et al.[7]. Rice bacterial blight isolates were subcultured on solid PSA medium (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, 20 g/L Bacto agar and pH 7.0). Three-month-old rice plants were inoculated with three *Xanthomonas oryzae* pv. *Oryzae* isolates of the Philippines, race 2 (PXO086), race 4 (PXO113) and race 6 (PXO99), and two *Xanthomonas oryzae* pv. *Oryzae* isolates of China, C4 (Z173) and C5 (GD1358), with a bacterial suspension with a density of 0.4—0.6 at OD_{600}. Ten fully expanded leaves from each mutant were used for inoculation experiments. Inoculated plants were maintained in a greenhouse and disease lesions were examined 2 weeks after inoculation.

2 Results

2.1 Isolation and genetic analysis of lesion mimic mutants

To date, we have generated around 7000 independent T-DNA tagged lines by the use of an *Agrobacterium*-mediated, highly efficient rice transformation procedure[16]. In the T_0 and T_1 generations, all these tagged lines were carefully examined. Ten lines were found to develop spontaneous lesions under

pathogen-free conditions in the T_1 generation (Fig. 1). However, mimic lesions were invisible on the leaves of these lines in the T0 generation. Moreover, as expected for a single recessive locus (Table 1), the wild-type and mutant plants were segregated at a ratio of approximately 3 ∶1 in the T_1 generation of these lines. Similar results were also obtained in the T_2 generation of heterozygous T_1 plants. These results demonstrated that each mutation was controlled by a recessive nuclear gene. These mutants were called lesion mimic mutants (*lm*), and tentatively designated as *lm1* — *lm10*, respectively. Among them, the mutant *lm10* was lethal (around 3 months after sowing), four others (*lm1* — *lm3*, *lm9*) were fully sterile, and the remaining five (*lm4* — *lm8*) were partially fertile. In addition, allelism tests revealed that the five partially fertile mutants were non-allelic (data not shown).

Fig. 1. Lesion mimic phenotypes and histochemical detection of *lm* mutants. (a) Lesion mimic phenotypes of *lm* mutants. The fifth leaf from 5-week-old plants of cv. Nipponbare and *lm* mutants were harvested. NB indicates Nipponbare. (b) Trypan blue staining at the sites of necrotic lesions of *lm* mutants. Leaf segments representative of lesion mimic phenotypes of *lm* mutants were used.

Table 1. Copy number analysis of T-DNA inserts in the T_0 generation and segregation ratios in the T_1 generation for ten *lm* mutants

Mutants	Copy No. of T-DNA inserts	Total No. of plants	No. of wild-type plants	No. of mutant plants	Ratio	χ_c^2	*P*
lm1	2	178	130	48	2.71	0.2690	0.50—0.75
lm2	1	120	93	27	3.44	0.2770	0.50—0.75
lm3	1	145	112	33	3.39	0.2780	0.50—0.75
lm4	3	204	152	52	2.92	0.0066	> 0.90
lm5	2	135	99	36	2.75	0.1208	0.50—0.75
lm6	1	144	105	39	2.69	0.3333	0.50—0.75
lm7	2	179	132	47	2.81	0.0912	0.75—0.90
lm8	1	201	155	46	3.36	0.3728	0.50—0.75
lm9	2	230	170	60	2.83	0.0927	0.75—0.90
lm10	1	389	295	94	3.14	0.1036	0.50—0.75

2.2 Lesion mimic phenotypes of *lm* mutants

Ten spotted leaf mutants were carefully investigated in the T_1 generation regarding the timing of lesion formation, size and color of lesions. Lesion mimic phenotypes on the mature leaves of these mutants are shown in Fig. 1 (a) and Table 2. Visible lesions

appeared at the early developmental stage (2 to 3 weeks after sowing) in *lm1*, *lm3*, *lm4*, *lm5*, *lm7* and *lm8*, while lesions were seen about 4 to 5 weeks after sowing in *lm2*, *lm6*, *lm9* and *lm10*. Lesion formation of *lm* mutants was developmentally regulated, with older leaves showing more lesions than younger leaves. Mutant *lm1* showed small (< 3 mm in diameter), yellowish-brown, and necrotic lesions. The mutant *lm2* initially exhibited large (> 10 mm long), yellow, and necrotic lesions at the tip region of leaves, and then these lesions became gray, which was similar to the phenotype of the inoculated rice plants with virulent bacterial blight isolates. Mutant *lm3* displayed medium-sized, discrete, reddish-brown, and necrotic lesions, which were densely and randomly distributed on fully expanded leaves. In *lm4*, large, yellow and necrotic lesions initially appeared at the leaf tip, and then expanded along the leaf margin. Both *lm5* and *lm6* displayed large, yellowish-brown and necrotic lesions. Compared with *lm5*, the initiation of lesions in *lm6* lagged behind obviously. In *lm7*, small, brown, and necrotic lesions were uniformly and densely distributed on fully expanded leaves. Mutant *lm8* showed medium-sized, brown, and necrotic lesions. Similar to *lm1*, the mutant *lm9* also displayed small yellowish-brown, and necrotic lesions. However, *lm1* was notably different from *lm9* with respect to the timing of lesion formation and plant height. In *lm10*, small, yellowish-brown, and necrotic lesions were uniformly spread over the entire leaf surface.

In addition, all the mutants showed stunted growth. The mutant *lm2*, the dwarfest of all, was only 42.4 cm tall. Even the tallest mutant *lm5* was only 84.5 cm in plant height. Compared to the wild-type plants, their plant height decreased by 61.2% and 22.4%, respectively.

Table 2. Phenotypes of isolated lesion mimic mutants

Mutant	Genetic data	Plant height[a]	Fertility	Lesion mimic phenotypes		
				Timing[b] (weeks)	Size[c]	Color
lm1	Recessive	Dwarf	Fully sterile	2—3	Small	Yellowish-brown
lm2	Recessive	Dwarf	Fully sterile	4—5	Large	Yellow
lm3	Recessive	Dwarf	Fully sterile	2—3	Medium	Reddish-brown
lm4	Recessive	Semi-dwarf	Partially fertile	2—3	Large	Yellow
lm5	Recessive	Semi-dwarf	Partially fertile	2—3	Large	Yellowish brown
lm6	Recessive	Semi-dwarf	Partially fertile	4—5	Large	Yellowish brown
lm7	Recessive	Semi-dwarf	Partially fertile	2—3	Small	Brown
lm8	Recessive	Semi-dwarf	Partially fertile	2—3	Medium	Brown
lm9	Recessive	Semi-dwarf	Fully sterile	4—5	Small	Yellowish brown
lm10	Recessive	Dwarf	Early lethality[d]	4—5	Small	Yellowish brown

a) Dwarf, < 50 cm; semi-dwarf, 70—85 cm
b) Timing indicates the number of weeks after sowing when lesions became visible
c) Small, < 3 mm in diameter; medium, 3—10 mm; large, > 10 mm
d) Indicates that fertility was not examined in *lm10* due to its early lethality

2.3 Confirmation of the tagged T_0 plants for 10 lesion mimic mutants

PCR amplification and Southern blot analysis were carried out to investigate the T-DNA insertion in the transgenic T_0 plants for 10 lesion mimic mutants. In PCR amplification, all of them showed specific bands of the *hpt* gene using primer pairs of HygF and HygR. The positive result was also confirmed by Southern analysis of T_0 tagged plants using the *hpt* gene fragment as a probe. Based on the T-DNA structure of pCAMBIA1301, genomic DNA of T_0 tagging plants was digested with *Hind* III for Southern analysis. The number of hybridizing bands reflected the copy number of integrated T-DNA in the rice plants. As shown in Table 1, the T_0 tagged plants for all these mutants carried one to three copies of T-DNA inserts, respectively.

2.4 Cosegregation analysis of lesion mimic phenotypes with T-DNA inserts

To investigate whether the lesion mimic mutations were from the T-DNA integration, PCR amplification was performed in the progeny of the tagged T_0 plants for 10 lesion mimic mutants. All T_1 individuals per mutant were used for cosegregation analysis. As a result, all the mutant T_1 plants of *lm6* displayed specific bands of the *hpt* gene (Fig. 2 (a)). The wild-type T_1 individuals of *lm6* were further subjected to PCR amplification using the same primer pair as above. As expected for a single copy of T-DNA inser-

tion (Table 1), approximately two-thirds of the wild-type T_1 plants of *lm6* showed desired amplified bands (Fig. 2 (b)). These results preliminarily show that this mutation is induced by T-DNA integration. For each of the other nine mutants, some of the T_1 mutant individuals produced no amplified bands, showing that the remaining nine mutations were not caused by T-DNA insertion.

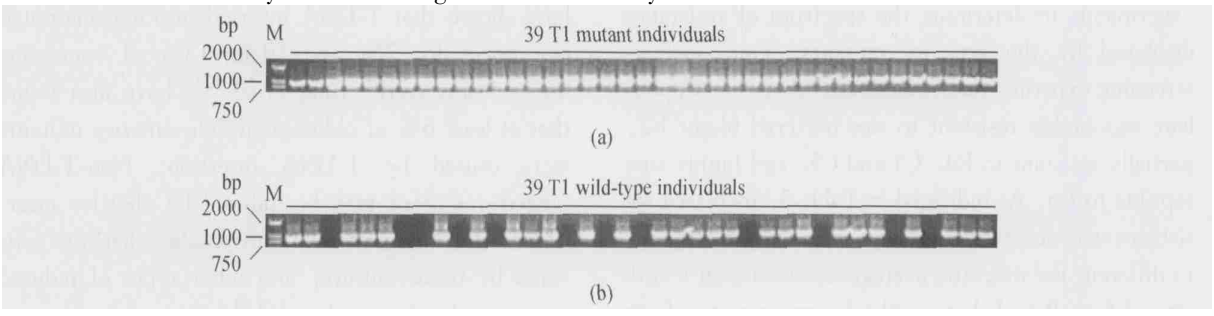

Fig. 2. Cosegregation analysis of the lesion mimic phenotype of *lm6* with T-DNA inserts. (a) PCR amplification of the mutant T_1 progeny of the T_0 transgenic plant for *lm6*. (b) PCR amplification of the wild-type T_1 progeny of the T_0 transgenic plant for mutant *lm6*. M, DL2000 DNA marker.

2.5 Histochemical detection of cell death in *lm* mutants

Trypan blue staining has widely been used to visualize dying cells and to evaluate cell membrane integrity. Since mimic lesions appeared on the leaves of each mutant at different developmental phases, two leaves per mutant were taken when lesions became visible. Subsequently, theses leaves were cut into segments 2 cm long for trypan blue staining. Leaf segments from 4-week-old plants of *lm1*, *lm3*, *lm4*, *lm5*, *lm7* and *lm8*, and 6-week-old plants of *lm2*, *lm6*, *lm9* and *lm10* were stained with trypan blue. Deep blue staining in cells at the site of necrosis in *lm* mutants represented localized cell death (Fig. 1 (b)). Blue staining was not observed in the young leaves (less than 2 weeks old) of *lm* mutants in which mimic lesions were not present. In the leaf segments of *lm5* and *lm9*, where large lesions developed, deep blue staining was localized on those newly senescent and dying cells within the edge of the necrotic lesions.

2.6 Resistance reaction of *lm* mutants to rice bacterial blight

To examine whether *lm* mutants displayed enhanced resistance to pathogen infection, a Philippline bacterial blight race 6 (R6) compatible with cv. Nipponbare was utilized to challenge nine *lm* mutants when mimic lesions appeared on their leaves. Since the mutants were from the same genetic background, one wild-type cv. Nipponbare corresponding to the background of the mutants was used as a control. Two weeks after inoculation, the symptoms on the mutants were compared with those on the wild-type control. Due to its early lethality, *lm10* was not chosen for the inoculation experiments. In *lm1*, *lm2* and *lm9*, the challenged plants normally died 1 to 2 weeks after inoculation. Relative to the wild-type control, the remaining six mutants showed slighter disease lesions, hence exhibiting enhanced resistance to this isolate (Fig. 3, Table 3).

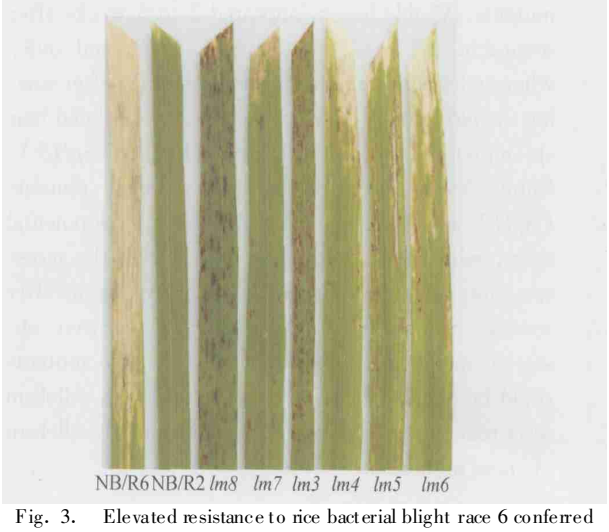

Fig. 3. Elevated resistance to rice bacterial blight race 6 conferred by six *lm* mutants. NB/R6 and NB/R2 are control plants (cv. Nipponbare) inoculated with race 6 and race 2, respectively. *lm3*—*lm8* were inoculated with race 6.

Table 3. Average disease lesion length (cm) on leaves of control plants and *lm* mutants in response to five different bacterial blight races

Race	Control	*lm3*	*lm4*	*lm5*	*lm6*	*lm7*	*lm8*
C4	1.2	0	0.8	0	0.5	0.3	0.4
C5	1.5	0	1.0	0.6	0.8	0.3	0.9
R2	0.5	0	0	0	0	0	0
R4	2.2	0	1.4	0.8	1.2	0	0.5
R6	10.6	0	2.4	1.4	4.1	0.5	1.5

Note: The average disease lesion length was calculated from 10 leaves 14 days after inoculation.

Two additional Philippine *Xanthomonas oryzae* pv. *oryzae* isolates, race 2 (R2) and race 4 (R4), and two Chinese *Xanthomonas oryzae* pv. *oryzae* isolates C4 and C5, were chosen for the inoculation experiments to determine the spectrum of resistance displayed by the six *lm* mutants. Our previous screening experiments demonstrated that cv. Nipponbare was highly resistant to rice bacterial blight R2, partially resistant to R4, C4 and C5, and highly susceptible to R6. As indicated in Table 3, increased resistance was observed in the six mutants. In response to different isolates, the average disease lesion length ranged from 0 to 4.1 cm in the *lm* mutants and from 0.5 to 10.6 cm in the wild-type plants (Table 3). Therefore, the six *lm* mutants exhibited the broad-spectrum resistance to rice bacterial blight.

3 Discussion

A large number of lesion mimic mutants have been found in rice. Takahashi et al.[6] analyzed three lesion mimic mutants (*cdr1*, *cdr2* and *Cdr3*). The *cdr1* and *cdr2* mutations were recessive, while *Cdr3* was dominant. Yin et al.[7] characterized nine *spl* mutants. Visible lesions appeared 2 to 3 weeks after sowing in *spl1*, *spl2*, *spl3*, *spl4*, *spl5*, and *spl9*, whereas lesions were seen around 3 months after sowing in *spl6* and *spl7*. Mizobuchi et al.[8] isolated four spl mutants (*Spl12*, *spl13*, *spl14* and *Spl15*). Mimic lesions occurred in the dominant mutants (*Spl12* and *Spl15*) from an early developmental stage, while lesion mimics were visible in the recessive mutations (*spl13* and *spl14*) 8—10 weeks after sowing. Since some similar characteristics were observed among *cdr*, *spl* and *lm* mutants, *lm* mutants could be allelic to *cdr* and *spl* mutations. Allelism tests remain to be performed to determine the allelism of these mutations.

Rice bacterial blight is one of destructive diseases in rice. Since many lesion mimic mutants in rice showed increased resistance to this pathogen, these mutants would be very useful for breeding of disease-resistant rice. In our inoculation experiments, six *lm* mutants showed enhanced resistance to rice bacterial blight when lesions were formed. Moreover, the *lm* mutants with stronger disease resistance showed stunted growth and some abnormal agronomic traits, which limits the practical use of them in breeding programs. In order for such mutations to be economically useful, the deleterious effects they cause must be minimized.

T-DNA tagging has widely been used for rice functional genomic analysis. However, a significant fraction of mutations were shown not being caused by T-DNA insertion. Jeon et al.[19] and Jeong et al.[20] have shown that T-DNA inserted into and disrupted rice genes for 7% and 10%—15% of transgenic lines, respectively. Jung et al.[21] have also found that at least 6% of chlorophyll-deficient rice mutants were caused by T-DNA insertion. Non-T-DNA tagged mutations may be induced by abortive insertion events[22], unknown transposable elements activated by tissue culture, and other types of induced mutations by tissue culture[23, 24]. In our experiment, out of 10 *lm* mutants, only one mutant *lm6* was preliminarily shown to segregate with the T-DNA insertion. The mutant *lm6* was partially fertile, and exhibited enhanced resistance to rice bacterial blight. Cloning of this lesion mimic gene would be helpful for revealing the molecular mechanism underlying cell death and disease resistance in rice.

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