Characterization of Lesion-Mimic Mutant Spotted Leaf 6 in Rice

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Abstract

Rice lesion-mimic mutants (LMM) exhibit spontaneous cell death in the absence of any pathogenic infection and induce various spots on the leaf surfaces and leaf blades. We studied the phenotypes of *bl1, bl2, spl3, spl4, spl5* and *spl6* LLM mutants of rice. Tiny spots were appeared at late tillering stage on the leaf of the mutants and their formation was developmentally controlled. Among them, one of the lesion mimic mutants spotted leaf 6 (*spl6*) was further characterized. Small and reddish-brown lesions were initiated at tillering stage and formed parallel lines on the leaf surfaces of the *spl6*. Using microscopy, damaged thylakoid membranes of mesophyll chloroplasts were seen in non-spotted sections and were absent in spotted sections of the mutant. Proteome analysis revealed, 159 protein spots were expressed differentially among mutants and wild type. However, protein disulfide isomerase, transketolase, thioredoxin peroxidase, ATP synthase, RuBisCO-L and RuBisCO-ACS were absent in the mutant but were abundant in the wild type. Absence of those proteins might be the cause of the failure to protect cells against oxidative burst, indicating the significant role of oxidative stress in lesion formation. Northern blot results revealed the significant correlation with proteome data.

Media summary

Understanding the molecular mechanism of lesion formation and cell death in rice leaves.

Key words

Spotted leaf, mutant, cell death, oxidative burst lesion mimic, proteomes

Introduction

Plants are exposed to various kinds of abiotic and biotic stresses as well as mechanical damages in their life cycle. Plants protect themselves from stresses by creating response against the stress elicitors through programmed cell death or spontaneous death of localized cell (Yamanouchi et al. 2002). Some mutant plants show genetically autonomous cell death without pathogenic attacks which are called lesion mimic mutants (Yin et al. 2000; Liu et al. 2004), which have been identified in rice (*Oryza sativa*) (Kiyosawa, 1970), and other crops (Greenberg and Ausubel. 1993). These lesion mimic mutants share a unique lesion phenotype with inducible expression of defense related genes (Mori et al., 2007; Tsunezuka et al., 2005). Rice lesion mimic mutant *spotted leaf 6* (*spl6*) form early lesion at tillering stage with tiny expansion but gradually increase into large lesion through continuous formation of new lesion that form

longitudinal line (Kang et al., 2007). No molecular and cellular characterization of *spl6* has been reported to date. As the proteome analysis is one of the direct approaches for finding functional proteins, it may provide clues to investigate the molecular mechanisms of lesion formation.

Methods

Eleven *spl* mutants rice and wild type rice were used for their phenotypic and molecular study. For TEM analysis ultra thin sections of wild type, non-spotted and spotted leaf of *spl6* were used and were observed under electron microscope. Proteins from matured leaves were isolated for proteomic analysis. Extracted proteins were analyzed by 2-D gel electrophoresis. Twenty five differentially expressed protein spots were analyzed using Ettan MALDI-TOF. Public data bases were used for the protein homology and annotation search. Several cDNAs were amplified by RT-PCR and purified, and were used for probe synthesis to use in Northern blot.

Results

The studied rice lesion mimic mutants were found forming spontaneous lesions on the leaf blades. Most cases there were no visible spots on the leaves at the seedling stage, but spots appeared at the tillering stage as tiny specks and became clearly visible within 60 d and became necrotic at the milk stage (Figure. 1.i.), indicating the developmental pattern of spot formation. Spot formation was propagation type where lesions initiated sparsely at an early developmental stage but expanded rapidly to the entire leaf blades (Figure. 1.i.).

Anatomical features of mesophyll cells of mutant showed that cells were dark brown with very few greenish parts indicating the death of the mesophyll cells and were found degradation of the chloroplasts in the mesophyll cells. Damaged chloroplasts with ruptured thylakoid membranes were found using transmission electron microscopy (TEM) analysis (Figure. 1.ii.) as well as plastoglobules were found deposited in the cytoplasm which might be due to the accumulation of chemical substances or callose (Figure. 1.ii.).

i. **ii.**

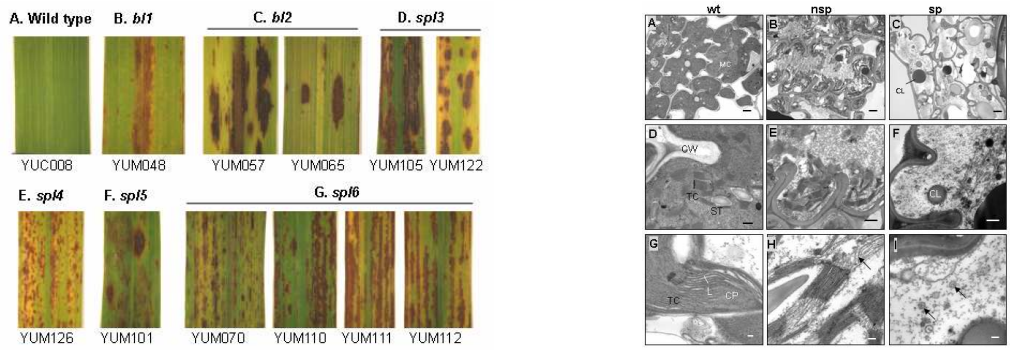

Figure 1. Phenotype of spl6 mutant leaf. **i.** Magnification of different *spl* mutant leaves. **A.** Wild type. **B.** *bl1*. **C.** *bl2*. **D.** *spl3*. **E.** *spl4*. **F.** *spl5*. **G.** *spl6*. YUM indicate the ID of mutant germplasm. **ii.** TEM analysis of wild type and *spl6* leaves. **A. B. & C.** Ultrastructure of chloroplasts of wild type (wt), non-spotted (nsp) and spotted (sp) leaf blades of *spl6* mutant. **D. E. & F.** Close-ups of wt, nsp and sp leaf chloroplast. **G. H. & I.** Close-ups of thylakoids. CL, callose; CP, chloroplast; CW, chloroplast wall; L, lamella; MC, mesophyll chloroplast; TC, thylakoids and ST, stroma. Arrows indicate broken lamella (H) and plastoglobules (I).Bars: A, B, C, 2 μM; D, 0.5 μM; E, 2 μM; F, 0.5 μM; G, 0.1 μM; H, 0.1 μM; I, 2 μM.

Proteomic analysis was carried out using *spl6* and wild type rice leaves. Representative images of 2-D gels indicate approximately 800 protein spots were reproducibly detected in gels. However, 159 spots were expressed differentially between mutant and wild type leaf proteins. Whereas, 114 protein spots were up-regulated and 45 spots were down-regulated. Ettan MALDI-TOF-MS analysis of the 25 spots, which displayed more than a two-fold variation, indicate that 9 annotated and two unknown proteins were down regulated and catalase was up regulated in the mutant (Figure. 2A, Table 1). Northern blot results demonstrate the significant correlation of transcriptions with those of translations in wild type and mutant (Figure. 2B).

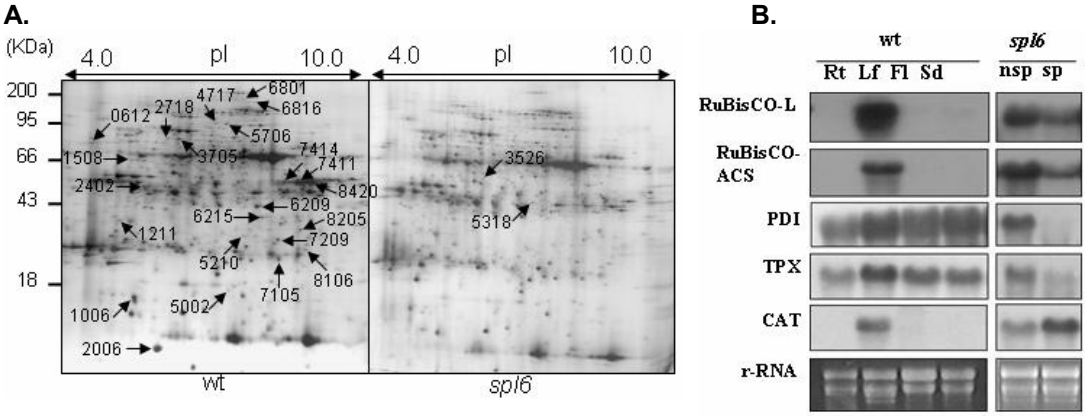

Figure 2. Proteome and Northern blot analysis. **A.** 2-DE gels of mutant leaf proteins. Arrows indicate more than two-fold up-regulated spots among wt and mutant. **B.** Expression of PDI, TPX, RuBisCO-L, CAT and RuBisCO-ACS at RNA level of wild type and mutant. Total RNAs were extracted from roots (rt), leaves (lf), flowers (fl) and seeds (sd) of wild type as well as spotted (sp) and non-spotted (nsp) leaves of mutant rice.

Table 1. List of selected proteins differentially expressed between wild type and *spl6* mutant

Protein type	Spot ID	Homologous protein	Accession	MM	pI	Quantity		No. of peptide	Coverage (%)
						wt	*spl6*		
Metabolism	0612	Protein disulfide isomerase	gi|7209794	72.48	4.40	821	0	16	31
	2006	Ribulose 1,5-bisphosphate carboxylase/oxygenase large chain	gi|11466795	7.23	5.06	8624	499	6	9
	1508	Transketolase	gi|50933551	60.81	4.82	384	0	7	9
Photosynthesis	1211	RuBisCO activase small isoform precursor	gi|8918361	31.16	4.69	414	0	18	24
Energy	2402	ATPase alpha subunit	gi|20143564	45.7	4.94	547	0	16	41
	5002	ATP synthase CF1 beta chain	gi|11466794	14.02	5.85	235	0	20	48
	6816	ATPase alpha subunit from chloroplast insertion	gi|37533324	113.1	6.45	71	0	7	12
	8205	Phosphoglycerate kinase	gi|50931897	27.97	7.51	532	0	8	27
Stress	1006	Thioredoxin peroxidase	gi|50252657	12.92	4.85	4690	0	6	30
	5318	Catalase 1 (CAT)	gi|6635734	44.1	5.90	20	245	11	18
Unknown	3526	OSJNBa0020P07.1	gi|32451277	54.74	5.29	3	131	7	12

Note: Spot ID; spot number given in figure 2A. Quantity; average proteins intensity of two analyses, MM; molecular masses, pI; isoelectric points, wt; wild type, Coverage %; percentage of sequence coverage.

Genetic study indicates that *spl6* gene is controlled by a single recessive gene and mapping of the *spl6*-linked SSR markers indicates two markers are nearest to the *spl6* (Figure 3).

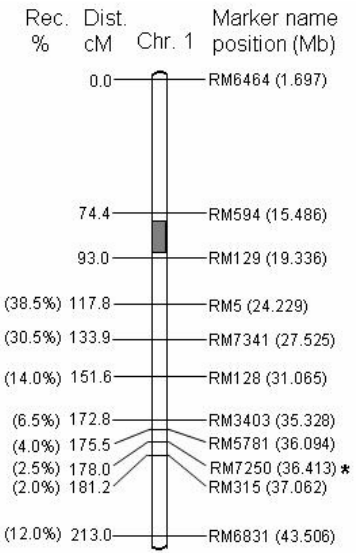

Figure 3. Mapping of *spl6*-linked SSR marker. * indicates *spl6* linked markers on rice chromosome 1. Rec. %; and Dits. cM; Recombination % and genetic distance in centi-Morgan, respectively.

Conclusion

Findings presented here suggest that reduction of several stress related genes expression in *spl6* might be the cause in failure to protect cells from ROS that causes oxidative burst resulting in thylakoid membrane degradation leading to programmed cell death and lesion formation in the mutant. Several physiological and biological changes, breakage of chloroplasts, rupture of cytoplasmic membranes and tonoplast, and degradation of cell organelles might take place in the mutant.

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