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The rice (*Oryza sativa*) *B*last *L*esion *M*imic Mutant, *blm*, may confer resistance to blast pathogens by triggering multiple defense-associated signaling pathways

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Abstract

Here we characterized a rice (*Oryza sativa* L.) *b*last *l*esion *m*imic (*blm*) mutant, identified previously in an *N*-methyl-*N*-nitrosourea-mutagenized population of the cultivar Hwacheong (wild type). The rice *blm* displayed spontaneous necrotic lesion formation on the leaves during development under long-day condition and temperature shift from 28 to 24 °C in the absence of obvious stress/disease, and provided us with a highly reproducible and convenient experimental system in the growth chamber to study *blm*. The *blm* phenotype resembled to the cell death of hypersensitive reaction (HR), and subsequent, two-dimensional gel electrophoresis (2-DGE) revealed induction of many leaf proteins; prominent among them were the three pathogenesis-related (PR) marker proteins of class 5 (one spot) and 10 (two spots). Interestingly, the rice *blm* manifested HR against all races tested of the rice blast fungus (*Magnaporthe grisea*), providing high resistance in a non-race specific manner. It was also observed that *blm* was highly resistant to hydrogen peroxide treatment. Using 2-DGE immunoblotting, we identified the presence of 4 new spots cross-reacting with a superoxide dismutase (SOD) antibody, only in *blm*, suggesting the expression of potentially new SOD protein (isoforms) during lesion formation. In the leaves of *blm*, autofluorescent compounds accumulated in and around the site of lesion progression. Moreover, enhanced levels of two major rice phytoalexins, sakuranetin and momilactone A were also observed in the leaves of *blm*. These results indicate that *blm* confers broad-spectrum resistance to multiple pathogens, and so, it could be hypothesized that the *BLM* gene product may control the HR-like cell death and its associated multiple defense signaling pathways, as evidenced by induction of known hallmark features (proteins/metabolites) linked with the defense responses, in rice.

Keywords: Blast pathogen; Lesion mimic mutant; *Oryza sativa*; Pathogenesis-related proteins and genes; Phenolics; Phytoalexins

Abbreviations: BLM, blast lesion mimic; CBB, coomassie brilliant blue; HR, hypersensitive reaction; H_2O_2, hydrogen peroxide; IEF, isoelectric focusing; LC–MS/MS, liquid chromatography tandem mass spectrometry; LMM, lesion mimic mutant; LSU, large subunit; MNU, *N*-methyl-*N*-nitrosourea; PR, pathogenesis-related; ROS, reactive oxygen species; RuBisCO, ribulose-1, 5-bisphosphate carboxylase/oxygenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSU, small subunit; SOD, superoxide dismutase; 2-DGE, two-dimensional gel electrophoresis.

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1. Introduction

Plants have developed complex signaling and defense mechanisms to protect themselves against invading pathogens. Recognition of the pathogen by the plant stimulates multiple signaling pathways leading to the activation of defense mechanisms, production of reactive oxygen species (ROS), cell wall fortification, and accumulation of phytoalexins, induction of pathogenesis-related (PR) genes, as well as systemic acquired resistance and hypersensitive reaction (HR) [23]. HR is regarded as the most efficient defense mechanism through rapid physiological changes arresting multiplication or expansion of the pathogens in the host cell [10,21,23,33]. HR, a well-programmed cell death phenomenon, is suggested to come from an interaction between the host resistant (R) gene and a corresponding avirulence (Avr) gene of pathogens, as demonstrated by the gene-for-gene theory [4,14,23].

HR or HR-like symptoms can also be activated in the absence of a pathogen, resulting in lesion appearance [9]. Lesions resemble either disease symptoms or pathogen-inducible HR. Plants capable of developing such spontaneous lesions are referred to as disease "lesion mimic mutants" (LMM), and have been identified in a variety of plants, such as maize [24], barley [50], rice [45,51], and *Arabidopsis* [11,20,21]. To date, 37 LMM have been identified in *Arabidopsis* [36]. LMM show different lesion phenotypes with respect to the timing and conditions of lesion appearance, and also the color and the size of the lesions [36]. These are classified into two types: one is an initiation type that forms small necrotic spots and another is feedback or propagation type that forms large necrosis similar to susceptible symptoms [49]. As many of the LMM present an aberrant regulation of cell death, enhanced resistance to pathogens and constitutive expression of defense mechanisms (autofluorescent phenolic compounds, callose deposition, ROS, PR marker proteins/genes, and phytoalexins), it is tempting to speculate that they are affected in genes of general importance for signaling pathways. Genes responsible for LMM phenotypes have been cloned in some plants [7,12,19,26].

Much effort has been exerted to reveal the underlying mechanisms for mutation(s) in the LMM. It has been suggested that LMM may affect mechanisms which control initiation or propagation of multiple biochemical events in pathogen-triggered R-gene specific resistance, or that, lesion mimic phenomenon may be the result of mutation, that alter cellular homeostasis as was shown in many transgenic plants [9]. Although many plant R genes against specific race of pathogens have been identified and characterized, the signaling network leading to defense response, such as HR or SAR still need to be analyzed. Elucidating how these LMM lead to spontaneous cell death along with significant resistance will provide clues to understand the regulation of cell death associated defense mechanisms against pathogen(s). Therefore, characterization of LMM is itself an important first step towards deciphering the defense mechanisms in plants.

Here we have characterized a unique lesion mimic phenomenon in one of the rice mutants mutagenized by *N*-methyl-*N*-nitrosourea (MNU), a chemical mutagen. Characterization of this LMM, called *b*last *l*esion *m*imic (*blm*) mutant [38] after its lesion resemblance with the blast fungal disease symptom, reveals first, spontaneous lesion formation with development and under long-day condition and temperature shift from 28 to 24 °C, second, resistance against fungal pathogens, and third, rapid and strong activation of a variety of hallmark features of defense mechanisms tightly linked with the stage of lesion progression in leaves.

2. Results

2.1. The rice blm mutant exhibits spontaneous necrotic lesions that are influenced by environmental signals

Previously, it was reported that one of the field-grown (Seoul National University, Seoul, Korea) rice breeding lines from cv. Hwacheong (wild type), mutagenized by MNU, exhibited necrotic lesions on leaves [38], while the mother line did not exhibit any symptom. The phenotype resembled the typical disease symptom caused by infection of the blast fungus, *Magnaporthe grisea*, and hence the mutant line was named *blm* [38]. Preliminary work revealed its race non-specific response to the blast pathogen *M. grisea*. Here, we characterize in detail the *blm* mutant, and focus more on its resistance to blast pathogens by triggering multiple defense-associated signaling pathways. Lesions started to appear around the tillering stage and reached to their maximum size (5.0 mm) at the heading stage (Fig. 1A) as also previously described [38]. Lesions were predominantly observed during long-day (LD) condition, however lesion development was strikingly attenuated in size and number under short-day condition, indicating that the lesion formation in the *blm* plant is highly dependent on environmental signals, such as day-length and temperature [38]. Other than lesion formation, the *blm* plants grew almost normally and set viable seeds. The *blm* seeds were in general slightly smaller in size to that of the wild type seeds (Fig. 1B). Taking into account the uniqueness of blast-like lesions in *blm*, it was reasoned that characterization of the *blm* mutant could provide insight into the signaling pathways linked with disease response(s). With this in mind, we established an in vitro system, and asked whether similar lesions can be produced under controlled light and temperature conditions. The *blm* and the wild type seeds were grown on 1/2 MS agar medium in sterilized glass bottles as described in Section 5. Lesions were first observed on the leaves of 1- to 2-week-old seedlings after a temperature shift from 28 to 24 °C 1-week after germination and that increased with time; the 3-week-old seedlings carried many lesions on leaves (Fig. 2A). The lesion was initiated as small chlorotic spot, similar to the susceptible lesion in early symptom by infection of *M. grisea*. The lesion further developed into a straw colored necrotic lesion or somewhat chlorotic lesion with orange or yellow colored center. No such symptom was seen in the leaves of the wild type. Notably, the lesion expan-