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Cloning and Expression of a Rice cDNA Encoding a *Lls1* Homologue of Maize

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A cDNA (*Oslls1*) encoding *Lls1*-homologue of maize was isolated from cDNA library of rice (*Oryza sativa* cv. Ilpum). The 2,138 bp of full length *Oslls1* clone contains an open reading frame of 1,623 nucleotides encoding 575 amino acid residues. The deduced amino acid sequence of *Oslls1* has a high level of homology with chlorophyll a oxygenases of *Arabidopsis thaliana* (67%) and *Marchantia polymorpha* (65%). Southern blot analysis of genomic DNA indicates the existance of a small gene family for *Oslls1* in the rice genome. The expression of *Oslls1* mRNA was induced in leaves and germinating seeds. Treatment of H_2O_2 significantly down-regulated *Oslls1* expression. The expression of *Oslls1* mRNA was constitutively down-regulated in the *blm*, a rice mutant exhibiting spontaneous necrotic lesions. These results suggest that this *Oslls1* gene may be involved in cell death mechanisms in the *blm* mutant of rice.

Keywords : *Lls1*, disease lesion mimic, chlorophyll a oxygenase, cell death mechanism.

Hypersensitive reaction (HR) is a localized response and characterized by occurrence of physiological changes resulting in plant cell death at the sites of attempted pathogen infection, and cessation of pathogen growth (Hammond-Kosack and Jones, 1996; Greenberg, 1997). Binding of avirulence gene product of the pathogen (elicitor) to resistance gene product of the plant (receptor) would stimulate a signaling cascades leading to the activation of defense mechanisms, such as production of active oxygen species (AOS), cell wall fortification, accumulation of salicylic acid (SA) and phytoalexin, and induction of pathogenesis-related (PR) genes (Hammond-Kosack and Jones, 1996) leading to HR (Frederick et al., 1998; Jones, 1997; Leister et al., 1996; Tang et al., 1996).

In certain instances, however, some of these defense mechanisms can be triggered in the absence of pathogens, resulting in lesion formation (Dangl et al., 1996). Lesions are similar in appearance to necrotic or HR lesion caused by a pathogen attack. These mutants are termed "disease lesion mimics", and they were observed in a variety of plants such as maize, barley, rice and Arabidopsis (Dietrich et al., 1994; Jorgensen, 1992; Takahashi et al., 1999; Ullstrup, 1967). Cell death of disease lesion mimic or programmed cell death in plants is an active process occurring in response to internal and external signals (Greenberg, 1996). Multicellular organisms maintain homeostasis by self-destruction of cells when they are no longer needed or if they are damaged (Ameisen, 1996). This is accomplished by activation of genetically regulated cell suicide machinery that requires the active participation of signaling pathways.

There are several examples of programmed cell death at specific sites and times in plants such as xylem and root cap differentiation (Kosslak et al., 1997; Wang et al., 1996), and senescence and death of mature leaves in deciduous plants (Bell, 1996; Goldberg et al., 1993). Although a variety of positive and negative regulator genes involved in the cell death mechanisms have been well characterized in mammalian systems (Nagata and Golstein, 1995; Steller, 1995; Thompson, 1995), little information is available on cell death mechanisms in plants. Recently, however, several genes involved in cell death mechanisms in plant are being elucidated (Büschges et al., 1997; Dietrich et al., 1997; Gray et al., 1997; Tanaka et al., 1997).

Rice *dad-1*, a human *dad-1* cDNA homologue, could rescue the temperature-sensitive *dad-1* mutant of hamster cells from apoptotic death, suggesting that function of the rice *dad-1* homologue is interchangeable for programmed cell death in mice (Tanaka et al., 1997). *Lls1* in maize and *Lsd1* in Arabidopsis are thought to function in the novel negative control mechanisms of cell death (Gray et al., 1997). Mutations in both genes result in spontaneous activation of cell death pathways in the absence of a pathogen. Recently, lesion mimic mutants have also been reported in rice (Takahashi et al., 1999). However, the biochemical mechanisms involved in spontaneous cell death remain to be elucidated.

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We recently reported the blast lesion mimic mutant (*blm*) of rice exhibiting spontaneous cell death under pathogen-free condition (Park et al., 2000). The phenotype of the *blm* mutant is similar to the maize *lls1* mutant in its lesion formation. Here we report isolation and expression of a cDNA gene encoding *Lls1* homologue in rice to understand the possible role on spontaneous lesion formation in the *blm*.

Materials and Methods

Plant materials and H_2O_2 treatment. The blast lesion mimic mutant (*blm*) of rice was acquired from rice cv. Hwacheong by treatment of N-methyl-N-nirtrosourea (MNU), as previously described (Cha et al., 1997). Rice plants (*Oryza sativa* L. cv. Hwacheong and the *blm* mutant) were grown in the greenhouse with a controlled temperature regime (20-30°C) under natural light condition. The leaves of cv. Hwacheong and the *blm* were harvested at 20 and 40 days after planting, directly put into liquid N_2 and stored at -80°C. For H_2O_2 treatment, rice leaves at 20 days old stage were cut and immersed in 50 mM H_2O_2 for 24 hr.

DNA isolation and polymerase chain reaction (PCR). Genomic DNA was extracted from rice leaves according to the method described by Rogers and Bendich (1985). Two PCR primers were designed from the expressed cDNA sequence of the rice which was available in the GenBank database under the accession number D46313 to amplify rice *Lls1* homologue. The primers used for PCR are as follows: DOX1, sense; 5′-CTGCAGCGCCGGAG-TCTGAACGTGTC-3′; antisense; DOX2, 5′-TGCAGCCGGA TGCGCACGTTGAGCA-3′. PCR reaction was performed with rice genomic DNA and *Taq* polymerase (TaKaRa Shuzo Co., Shiga, Japan) on a Perkin-Elmer 9600 PCR thermocycler (PE Applied Biosystems, Foster City, CA, USA) using the following cycling parameters; 5 min at 94°C, followed by 35 cycles (2 min at 60°C, 3 min at 72°C, 1 min at 94°C) with 3.5 min at 60°C and at 72°C for 7 min. PCR product was cloned using the pGEM-T Easy Vector system (Progmega, Madison, WI, USA).

cDNA library screening and sequencing of *Oslls1*. For cloning of a full size cDNA clone, cDNA library of cv. Ilpum was screened with PCR product as a probe. Nucleotide sequences of three positive clones were determined using a BigDye DNA sequencing kit (Perkin-Elmer Corp., Norwalk, CT, USA) on an ABI377 DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). All sequencing data were analyzed using Genetyx software (Software Development, Tokyo, Japan). Homology searches of nucleotide and amino acid sequences were performed using a BLAST in the GenBank and EMBL databases.

Southern blot analysis. Aliquots of 1 ug of purified genomic DNA were digested with restriction endonucleases, size fractionated in 0.8% agarose gel, and transferred onto Hybond N^+ membrane (Amersham Phamacia Biotech, Buckinghamshire, UK). The blots were hybridized with the ^{32}P-labeled *Oslls1* cDNA probe in hybridization solution (6X SSC, 5X Denhardts solution, 0.1% SDS and 50 mM phosphate buffer, pH 6.6) at 65°C for 24 hr. After hybridization, the blot was washed twice with 2X SSC and 0.1% SDS for 10 min each at room temperature, and twice with 0.2X SSC and 0.1% SDS at 65°C for 10 min each. Then the membrane was exposed to X-ray film (Agfa-Gevaert, Germany) at -70°C.

Northern blot analysis. Total RNA was extracted from rice leaves using a LiCl method described by Prescott et al. (1987). Fifteen ug of total RNA was fractionated on 1.5% formaldehyde agarose gel and transferred onto Hybond N^+ membrane (Amersham Phamacia Biotech, Buckinghamshire, UK). Equal amount of RNA loading was confirmed by ethidium bromide staining of rRNAs. The blots were hybridized with the ^{32}P-labeled *Oslls1* cDNA probe in hybridization solution (50% Formamide, 5X SSPE, 0.5X Denhardt solution, 0.2% SDS with 0.1 mg/ml salmon sperm DNA) at 42°C. After hybridization, the blots were washed twice with 2X SSC and 0.1% SDS for 10 min each at room temperature, and twice with 0.2X SSC and 0.1% SDS at 45°C for 10 min each. Then the membranes were exposed to X-ray film (Agfa-Gevaert, Germany) with intensifying screens at -70°C.

Results and Discussion

Isolation and sequence analysis of a rice cDNA (*Oslls1*) encoding a maize *Lls1* homologue. To isolate *Lls1*-homologue gene in rice, two primers were designed based on the EST sequence from the GenBank (D46313). Nucleotide sequence of 559 bp PCR product was identical to corresponding region of the EST sequence, but it contained two introns, 95 bp and 140 bp, respectively. Three positive cDNA clones were isolated by screening of the cDNA library of cv. Ilpum with ^{32}P-labelled PCR product as a probe. A full length cDNA (*Oslls1*) was identified and further characterized by sequencing analysis.

Nucleotide and deduced amino acid sequence of the *Oslls1* gene is shown in Fig. 1. The *Oslls1* cDNA was 2,138 bp long and contained an open reading frame of 1,623 bp encoding 575 amino acids. A putative polyadenylation signal was found in the 3′ non-coding region. The predicted amino acid of *Oslls1* was used to search homologous sequences in the GenBank and EMBL databases using a BLAST. Significant sequence homology was found between the predicted Oslls1 protein and chlorophyll a oxygenases from *Arabidopsis thaliana* (67%), *Marchantia polymorpha* (65%), *Chlamydomonas reinhardtii* (39%), *Dunaliella salina* (40%) and *Prochloron didemni* (51%). Consensus amino acids in two conserved motifs found in chlorophyll a oxygenases and Lls1 of maize, rieske center-binding site and mononuclear iron binding site, were also found in Oslls1 (Fig. 2). However, low level of sequence homology was observed between Oslls1 and Lls1 of maize (14%).

On the other hand, according to supposition that maize *Lls1* encodes dioxygenase, an aromatic ring hydroxylating enzyme, repressible activity of cell death by *Lls1* product is thought to be mediated by detoxification of a phenolic com-

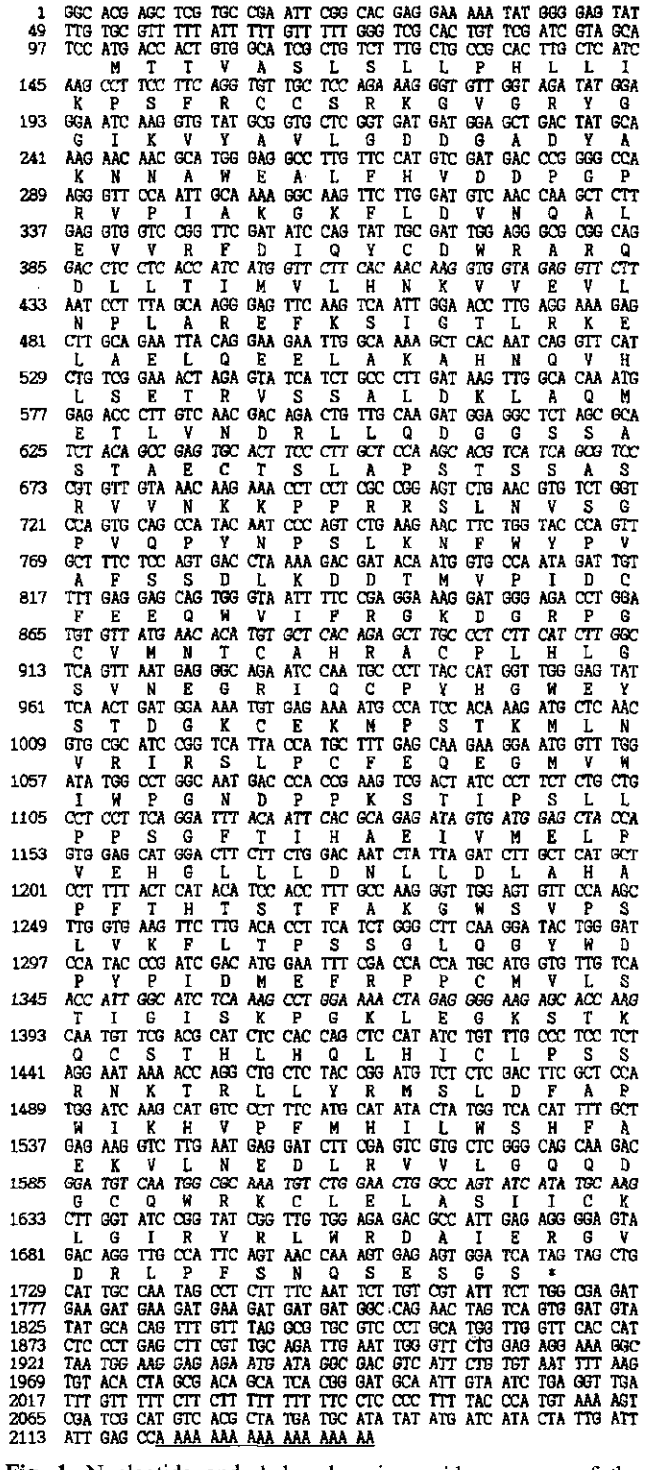

Fig. 1. Nucleotide and deduced amino acid sequence of the *Oslls1* gene (GenBank accession no. AF284781). Start and stop codons are indicated by an asterisk. A putative polyadenylation site is underlined.

pound (Gray et al., 1997). Since the existence of *Lls1* homologues in several mono- and dicotyledonous plants have been identified by sequence search in the GenBank,

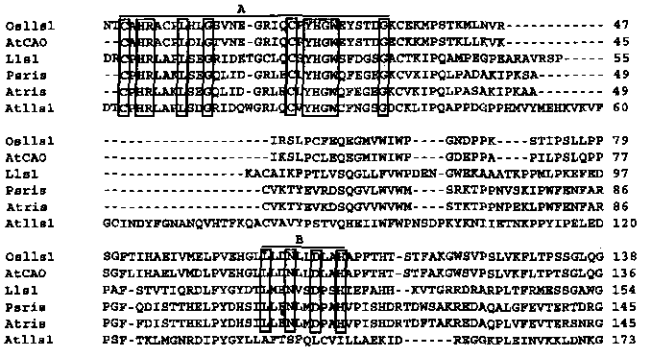

Fig. 2. Multi-alignment of amino acid sequences with several Lls1 homologues from other plant species. Atris, a putative rieske iron-sulfur protein in *Arabidopsis thaliana* (AC006585); Psris, a rieske iron-sulfur protein Tic55 in *Pisum sativum* (AJ000520); AtCAO, a chlorophyll a oxygenase in *Arabidopsis thaliana* (AB-030565); Lls1, a lethal leaf spot 1 in *Zea mays* (U77346); Atlls1, a lethal leaf spot 1 homolog in *Arabidopsis thaliana* (U77347). Gaps introduced for the alignment are indicated by hyphens. Identical amino acids in two conserved binding domains, rieske center-binding site (A) and mononuclear iron binding site (B), are boxed.

the *Lls1* homologue was suggested to have an important role in cell death mechanisms common in plants (Gray et al., 1997). Taking together, it is tempting to suggest the possibility of dual function of *Lls1*, one as chlorophyll a oxygenase and the other as a negative regulator of cell death.

Genomic organization of *Oslls1*. To understand genomic organization of the *Oslls1*, genomic Southern blot analysis was carried out using *Oslls1* cDNA as a probe.

Several hybridization bands were observed in most digestions, suggesting that *Oslls1* consists of a small gene family in the genome (Fig. 3). To reveal if there is a polymorphism of the *Oslls1* among different rice cultivars, Southern blot analysis was performed with genomic DNAs from several *japonica* and *indica* rice cultivas. As shown in Fig. 3, no polymorphism of *Oslls1* was detected among different rice cultivars.

Develpmental and tissue specific expression of *Oslls1* mRNA. To understand the regulation of *Oslls1* mRNA in different tissues, total RNA was extracted from leaves, sheath, roots and germinating seedlings and used for Northern blot analysis. Expression of *Oslls1* mRNA was strongly induced in leaves, and followed by germinating seedlings. No expression was detected in sheath and root samples (Fig. 4).

To understand the possible role of the *Oslls1* in spontaneous cell death in the *blm*, Northern blot analysis was performed using total RNAs from cv. Hwacheong and the *blm* mutant. Spontaneous lesions in the *blm* appear mainly near the tip of the oldest leaf at 40 days (7-8th leaf stage) after planting (Park, et al., 2000). High level of expression of *Oslls1* mRNA was detected at 20 days after planting in cv.

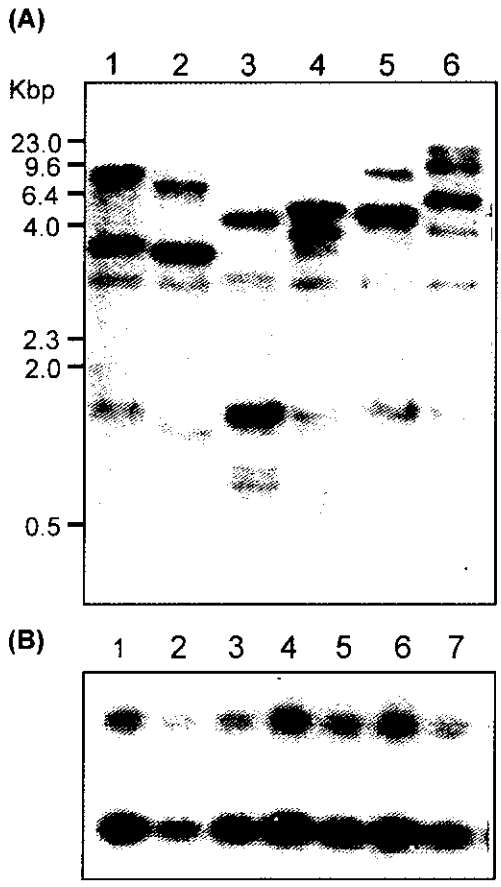

Fig. 3. Genomic Southern blot analyses of the *Oslls1* gene. (A) Genomic DNA from cv. Nipponbare was digested, blotted, and probed with ^{32}P-labelled *Oslls1* cDNA. Lane 1, *Eco* RI; lane 2, *Eco* RV; lane 3, *Hind* III; lane 4, *Sac* I; lane 5, *Xho* I; lane 6, *Xba* I. (B) Genomic DNAs from several rice cultivars were digested with *Eco* RI, blotted, and probed with ^{32}P-labelled *Oslls1* cDNA. Lane 1, Nipponbare; lane 2, Shimokita; lane 3, Touhoku IR9; lane 4, Tjina; lane 5, Milek Kuning; lane 6, Tadukan; lane 7, Kasalath.

Hwacheong, but the expression was strongly down regulated at 7-8th leaf stage. In the *blm*, however, expression level of *Oslls1* mRNA was much lower than cv. Hwacheong at 3-4th leaf stage and remained until 7-8th leaf stage without a little change.

In maize, however, the expression pattern of *Lls1* mRNA in the *lls1* mutant was essentially the same as in wild-type maize. Therefore, the phenotype of *lls1* mutant is not caused by abolished transcription but may be by interruption in the translation of a functional product (Gray et al., 1997). Since the expression of *Oslls1* mRNA was significantly down-regulated in the *blm* compared to that in wild type, *Oslls1* may be related with phenotype of the *blm* at the transcription level. On the other hand, since nucleotide sequence of *Oslls1* is similar to chlorophyll a oxygenase, down regulation of *Oslls1* in the *blm* may cause abnormal ratio of chlorophyll a and b conversion. This abnormal ratio

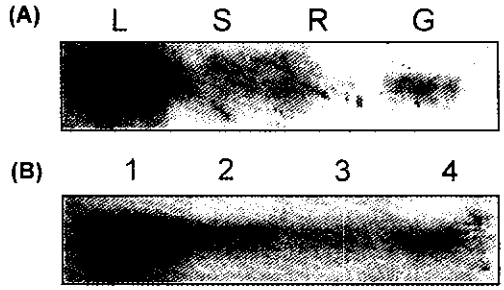

Fig. 4. Northern blot analyses of the *Oslls1* gene. (**A**) Expression patterns in different rice organs. Total RNA was extracted from leaf (L), stem (S), root (R) and germinating seeds (G). (**B**) Expression patterns of the *Oslls1* gene in cv. Hwacheong and the *blm* mutant. Total RNA was extracted from 20 days old plants of cv. Hwacheong (lane 1) and the *blm* (lane 2). Total RNA was also extracted from 20 days old plants of cv. Hwacheong (lane 3) and the *blm* (lane 4) after 50 mM H_2O_2 treatment. Fifteen ug of total RNA was loaded into each lane. The blot was hybridized with ^{32}P-labelled *Oslls1* cDNA. Equal amount loading of RNA was confirmed by Ethidium bromide staining of the gel.

between chlorophyll a and b may have a role(s) on developing spontaneous lesions in the *blm*.

Induction of *Oslls1* mRNA in response to exogenous H_2O_2. In response to exogenous application of 50 mM H_2O_2, cv. Hwacheong showed many dark brown spots on the leaves without any bleaching. However, no visible lesion appeared in the *blm* when treated with 50 mM H_2O_2 (Park et al., 2000). It was suggested that mutation in the *blm* induced a significant resistance to high concentration of H_2O_2. To examine the effect of H_2O_2 on the expression of *Oslls1* mRNA, total RNAs were extracted from leaves treated with 50 mM H_2O_2 in both cv. Hwacheong and the *blm* and used for Northern blot analysis. The expression level of *Oslls1* mRNA was significantly decreased in cv. Hwacheong when treated with H_2O_2, while that of the *blm* remained constant even after H_2O_2 application (Fig. 4B). These results suggest that down regulation of *Oslls1* is related to the gain of function on resistance to high concentration of H_2O_2. This is supported by the fact that peroxidase gene was constitutively activated in the *blm*, but induced by H_2O_2 treatment in cv. Hwacheong (unpublished data). Although the precise function of the *Oslls1* on lesion mimic phenomenon is not known at the moment, further analysis with other defense-related genes in the *blm* would provide clues for the understanding of R-gene specific as well as R-gene non-specific disease resistance in rice.

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