Bull. Fac. Life Env. Sci. Shimane Univ., 14：3－8, September 30, 2009

Role of tryptamine accumulation and DNA fragmentation in light-induced resistance of Sekiguchi lesion mutant of rice infected with *Bipolaris oryzae*

Makoto UENO*, Yumiko KUMURA, Junichi KIHARA, Sakae ARASE

Abstract Rice cultivar Sekiguchi-asahi was identified as a lesion mimic mutant derived from cv. Asahi. Sekiguchi-asahi showed light-enhanced resistance to *Bipolaris oryzae* infection inducing the formation of Sekiguchi lesions and tryptamine accumulation. No sporulation was observed on the Sekiguchi lesions. Furthermore, significant DNA fragmentation was observed in leaves with Sekiguchi lesions after inoculation with *B. oryzae* under light, as compared with brown spot lesions in the dark. However, pretreatment with 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) suppressed the formation of Sekiguchi lesions, tryptamine accumulation, and DNA fragmentation in Sekiguchi-asahi even under light. Light-induced resistance of rice against *B. oryzae* is supported by tryptamine with antifungal activity, and increased DNA fragmentation might contribute to enhance the accumulation of tryptamine involved in inhibition of pathogen development such as sporulation.

Keywords: Sekiguchi lesion・Rice・Tryptamine・DNA fragmentation・*Bipolaris oryzae*

Introduction

It is well known that spontaneous and artificial mutations in plants may induce the formation of lesion-like symptoms in tissues in the absence of pathogens (Dangl et al., 1996; Ryals et al., 1996). Such mutant plants are called lesion mimic mutants and 2 phenotypes are known in mutants: one is the initiation type characterized by the formation of small necrotic spots and the other is the propagation type characterized by the formation of large necrosis.

The rice cultivar Sekiguchi-asahi is a propagation-type lesion mimic mutant derived from cv. Asahi in Japan. This mutant shows two responses to *Magnaporthe grisea* infection by light treatments. Under visible light (400-700 nm), the formation of Sekiguchi lesions was induced by infection with *M. grisea*, whereas under UV light (290-400 nm) or in the dark, blast lesions or necrotic spots were induced (Arase et al., 1997). When Sekiguchi lesions were formed, the growth of infection hyphae was poor and spore formation was not observed. From these results, we concluded that the formation of Sekiguchi lesions is one of the basic indications of light-enhanced resistance in the rice cv. Sekiguchi-asahi and that visible light plays an important role in the expression of resistance (Arase et al., 2000a, b). Furthermore, the indole alkaloid compound tryptamine, a key factor in this light-enhanced resistance, was isolated from the Sekiguchi lesions (Arase et al., 2001). Tryptamine inhibited spore germination and appressorium formation of *M. grisea* at high concentrations (>600 μg/ml) and the growth of infection hyphae at low concentrations (150-300 μg/ml) (Arase et al., 2001). Moreover, we have reported that tryptamine-mediated DNA fragmentation is observed in leaves with Sekiguchi lesions by the inoculation with *M. grisea* under light, but not in leaves without Sekiguchi lesions in the dark (Ueno et al., 2004, 2008).

Further, Arase et al. (2000a) reported that the formation of Sekigchi lesions was also light-dependently induced by inoculation with *Bipolaris oryzae*. Furthermore, we indicated that tryptamine inhibited the spore germination and growth of infectious hyphae of *B. oryzae* at a concentration of over 150 μg/ml (Ueno et al., 2005).

Laboratory of Plant Pathology, Faculty of Life and Environmental Science, Shimane University, Matsue 690-8504, Japan.
*To whom correspondence should be addressed.

In the present paper, the role of tryptamine accumulation and DNA fragmentation on light-induced resistance in cv. Sekiguchi-asahi infected by *B. oryzae* is reported.

Materials and Methods

Plant and fungus

The rice cv. Sekiguchi-asahi (with resistance gene *Pi-a*) was used in this study. This cultivar was grown to the 6-7 leaf stage in a glasshouse, as described by Arase et al. (2000b). *B. oryzae* (Strain D6) was used as the pathogen. *B. oryzae* was cultured in potato sucrose agar (PSA) medium for 10 days at 26℃ and the spores were collected. These spores were used as the inocula.

Inoculation and light irradiation

Detached leaves of rice cv. Sekiguchi-asahi at the 6-7 leaf stage were inoculated with *B. oryzae* spores (10^5 spores/ml). The control leaves were sprayed with distilled water (DW). The spore-inoculated and the DW-treated leaves were kept in moist plastic cases covered with a plastic film for light treatment or aluminum foil for dark treatment, respectively, and irradiated by 40-W daylight fluorescent lamps (FLR40SW, Mitsubishi Co., Yokohama, Japan) suspended above the plastic cases. Under these filters, the light intensities on the leaf blades were adjusted to 405 $\mu W/cm^2$ by changing the distance between the lamps and the leaves. The light intensity was measured using a thermopile with a quartz window (MIR-100q, Mitsubishi Yuka Co. Ltd., Tokyo, Japan). After 72 h, the spore-inoculated and the DW-treated leaves were harvested for Sekiguchi lesion, tryptamine content, and DNA fragmentation analyses.

Chemical treatment

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) (Lancaster Synthesis, Ward, Hill, MA, USA) was used as a photosynthetic inhibitor. Detached leaf blades from 6-7-leaf-stage plants were immersed in DCMU solution at 25 μM for 24 h, inoculated with a spore suspension (10^5 spores/ml) of *B. oryzae*, and then maintained in moist plastic cases irradiated by 40-W daylight fluorescent lamps (FLR40SW, Mitsubishi Co., Yokohama, Japan) at 26 ℃. After 72 h, these detached leaves were used for the analyses of the formation of Sekiguchi lesions, tryptamine accumulation, and DNA fragmentation.

Extraction of tryptamine

Detached leaves of the rice cv. Sekiguchi-asahi was inoculated with *B. oryzae* or sprayed with distilled water. These rice leaves were kept under light or dark conditions for 0, 12, 24, 48, and 72 h. The leaves were extracted with 80% ethanol, and ethanol was evaporated at 40℃ under reduced pressure. The volume of the aqueous solution was adjusted to the initial weight of the leaves. Ten μl of the aqueous solution and the authentic tryptamine solution were spotted onto silica gel thin-layer chromatography (TLC) plates (Silica gel 60, Merck AG, Darmstadt, Germany) and then developed using an *n*-BuOH, AcOH, H_2O (4 : 1 : 1, v/v) solvent system. After development, the TLC plates were sprayed with ninhydrin solution and then heated at 100℃.

To analyze tryptamine accumulation per gram fresh weight, *B.oryzae*-infected leaves maintained under light or dark conditions for 72 h were extracted with 80% ethanol. The solution thus obtained was adjusted to pH 10.7 by adding 0.1 N Na_2CO_3, and then extracted with ethyl acetate (EtOAc). The EtOAc extracts were dissolved in methanol and subjected to high-performance liquid chromatography (HPLC) (Hitachi 1600, Wako Wakosil-Ⅱ 5C18AR, column size: 4.6×250 mm) and eluted with methanol : ammonium acetate (8 : 2, v/v), monitoring at 281 nm.

DNA extraction and gel analysis

Rice genomic DNA was extracted 72 h after inoculation with *B. oryzae* spores using the cetyltrimethylammonium bromide (CTAB) method described previously (Tanaka et al. 2001). Leaf blades (1 g) were ground with liquid nitrogen into a fine powder. Two milliliters of 2% CTAB DNA extraction buffer [containing 2% CTAB, 1.4 M NaCl, 0.1 M Tris-HCl (pH 8.0), and 20 mM ethylenediaminetetraacetic acid (EDTA)] was added to each ground sample and then incubated for 30 min at 65℃. After centrifugation at 18000 ×g for 15 min, the supernatant was collected and 1/10 of the volume of 3 M sodium acetate (pH 5.2) and an equal volume of phenol/chloroform were added to it. After further centrifugation at 18000

×g for 15 min, an equal volume of isopropyl alcohol was added to the aqueous phase and the solution was centrifuged at 18000 ×g for 15 min. Thereafter, the pellet was washed in 70% cold ethanol, dried and dissolved in Tris-EDTA (TE), and treated with RNase (100 μg/ml) for 30 min at 37℃. DNA was finally re-extracted using phenol/chloroform, precipitated with isopropyl alcohol, and resuspended in TE buffer. DNA was quantified by measuring absorbance at 260 and 280 nm. To detect the purified DNA fragments, the samples were examined on 2% (w/v) agarose gels and stained with a final concentration of 0.5 μg/ml ethidium bromide. DNA was photographed by an AE 6905 H Image Saver HR (ATTO, Tokyo, Japan).

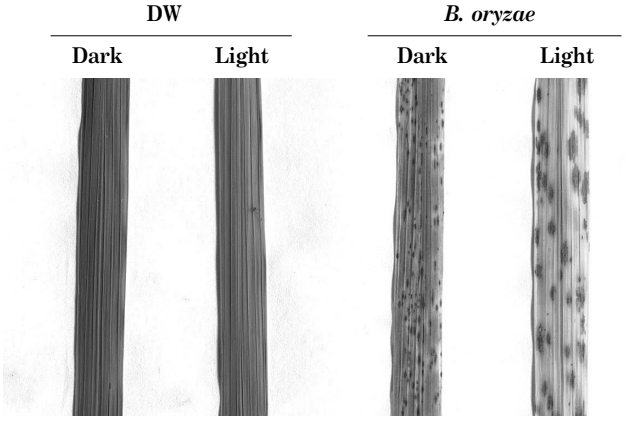

Fig. 1. Light-dependent Sekiguchi lesion formation in Sekiguchi lesion mutant infected with *Bipolaris oryzae*. Detached rice leaves were inoculated with *B. oryzae*. As a control, the other leaves were sprayed with distilled water (DW). Inoculated and non-inoculated leaves were kept under light or in the dark at 26 ℃.

Results

Light-dependent accumulation of tryptamine in leaves of rice cv. Sekiguchi-asahi infected with *B. oryzae*

In *B. oryzae*-infected leaves, Sekiguchi lesion formation was induced under light, whereas brown spot lesion formation was induced in the dark (Fig. 1). In DW-sprayed

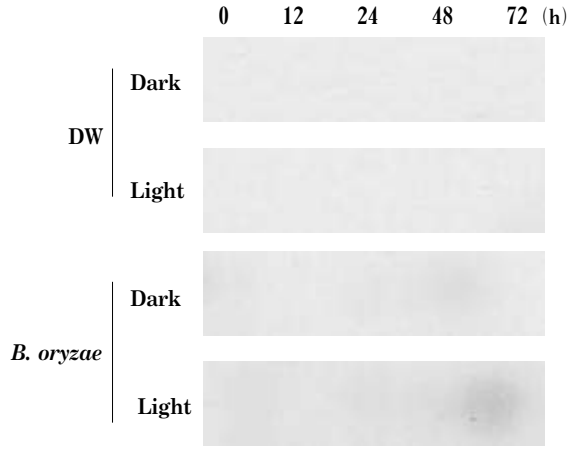

Fig. 3. Time course of tryptamine accumulation in Sekiguchi lesion mutant infected with *Bipolaris oryzae*. Detached rice leaves were inoculated with *B. oryzae*. As a control, the other leaves were sprayed with distilled water (DW). Inoculated and non-inoculated leaves were kept under light or in the dark at 26 ℃ for 0, 12, 24, 48, and 72 h. Tryptamine was extracted from inoculated and non-inoculated leaves and tryptamine was detected as a ninhydrin positive spot on the TLC plates.

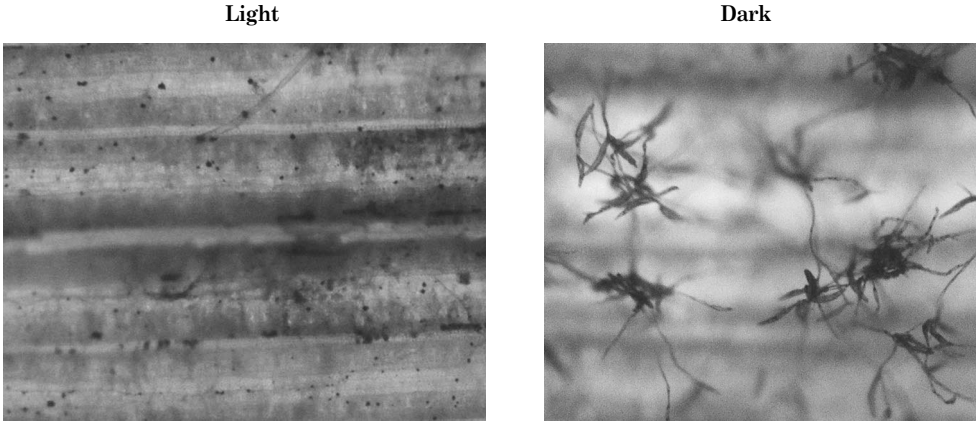

Fig. 2. Effect of light on sporulation in Sekiguchi lesion mutant infected with *Bipolaris oryzae*. The leaves of the Sekigucihi lesion mutant were inoculated with a spore suspension of *B. oryzae*, and kept under light (Light) and dark (Dark) conditions, respectively. Sporulation was investigated 5 days after inoculation.

leaves, however, any type of lesions was not induced, irrespective of the light conditions. When both the Sekiguchi and brown spot lesions were kept in a moist chamber, sporulation was observed on the brown spot lesion, but not on the Sekiguchi lesion (Fig. 2). To elucidate the relationship between tryptamine accumulation and the formation of Sekiguchi lesions, the amount of tryptamine accumulating in *B. oryzae*-infected leaves was investigated at 0, 12, 24, 48, and 72 h after inoculation. A large amount of tryptamine accumulated in the *B. oryzae*-infected leaves with Sekiguchi lesions under light, whereas tryptamine accumulation was not observed in the leaves with brown spots in the dark (Fig. 3).

Induction of DNA fragmentation in leaves of the rice cv. Sekiguchi-asahi infected with *B. oryzae*

In cv. Sekiguchi-asahi-*M. grisea* interaction, the importance of DNA fragmentation in Sekiguchi lesion formation, which is a marker of light-enhanced resistance, is demonstrated clearly (Ueno et al., 2004). To elucidate the relationship between Sekiguchi lesion formation and DNA fragmentation in *B. oryzae* infection, DNA fragmentation was investigated using leaves with Sekiguchi lesions or brown

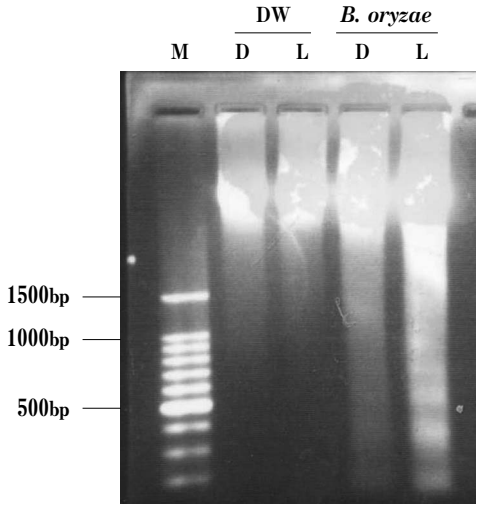

Fig. 4. Detection of DNA fragmentation in Sekiguchi lesion mutant inoculated with *Bipolaris oryzae*. Detached rice leaves were inoculated with *B. oryzae*. As a control, the other leaves were sprayed with distilled water (DW). Inoculated and non-inoculated leaves were kept under light (L) or in the dark (D) at 26 ℃ for 72 h. DNA was extracted from leaves by the cetyltrimethyl-ammonium bromide method, and 10 μg of DNA was loaded onto a 2% agarose gel. DNA fragmentation was observed by ethidium bromide staining.

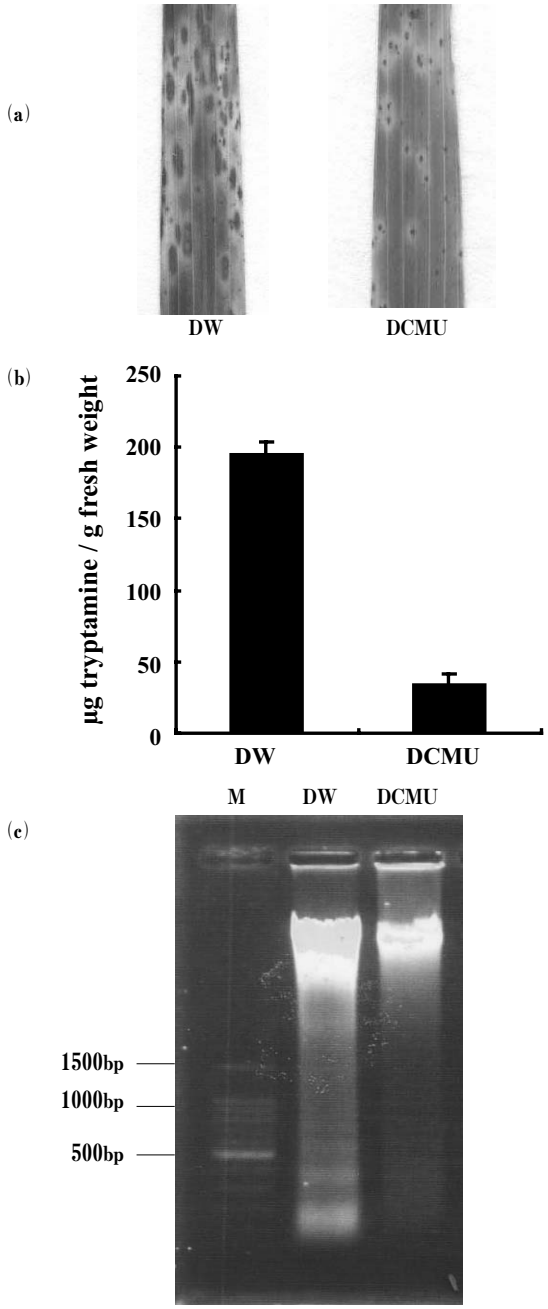

Fig. 5. Effect of 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) on Sekiguchi lesion formation (a), tryptamine accumulation (b) and DNA fragmentation (c) in Sekiguchi lesion mutant infected with *Bipolaris oryzae*. Leaves pretreated with DCMU or distilled water for 24 h were inoculated with *B. oryzae* and kept under light at 26℃ for 72 h. DNA was extracted from leaves by the cetyltrimethyl-ammonium bromide method, and 10 μg of DNA was loaded onto a 2% agarose gel. DNA fragmentation was observed by ethidium bromide staining.

spot lesions. As shown in Fig. 4, although DNA fragmentation was observed in leaves with both types of lesions, there was a difference in degree of fragmentation between light treatments. Under light, DNA fragmentation was significantly induced, as compared to that in the dark.

Effects of DCMU on the formation of Sekiguchi lesions, tryptamine accumulation, and DNA fragmentation in rice cv. Sekiguchi-asahi infected with *B. oryzae*

It is reported that photosynthesis is playing an important role in light-induced resistance in a mutant to *M. grisea* infection (Imaoka et al., 2008). In the DW-pretreated leaves, the formation of Sekiguchi lesions was light-dependently induced, whereas brown spot lesion formation was induced in the DCMU-pretreated leaves even under light as well as that in the dark (Fig. 5a). DCMU pretreatment inhibited tryptamine accumulation. According to HPLC analysis, the amount of tryptamine in DW-pretreated leaves with Sekiguchi lesions was 195.8 ± 8.0 μg/g fresh weight, while that in DCMU-pretreated with brown spots was 34.1 ± 7.3 μg/ g fresh weight (Fig. 5b). Furthermore, DCMU pretreatment inhibited DNA fragmentation in leaves inoculated with *B. oryzae* even under light (Fig. 5c).

Discussion

In a previous study, we reported the formation of Sekiguchi lesions, tryptamine accumulation, and DNA fragmentation in rice cv. Sekiguchi-asahi inoculated with *M. grisea* were significantly enhanced under visible light (400-650 nm), but not under near-UV light (300-400 nm) or in the dark (Ueno et al., 2004, 2007). Our studies demonstrated that cv. Sekiguchi-asahi shows tryptamine pathway-mediated resistance to *M. grisea* infection under light. Sekigchi lesion formation was also light-dependently induced by inoculation with *B. oryzae* (Arase et al., 2000a). In this study, sporulation of *B. oryzae* was observed in brown spot lesion induced in the dark, but not in the Sekiguchi lesion induced under light. No sporulation was also observed in *M. grisea*-induced Sekiguchi lesions. These results suggested that the Sekiguchi lesion formation showed the expression of light-induced resistance of rice against necrotrophic fungus, *B. oryzae* and semi-biotrophic fungus, *M. grisea*.

In *M. grisea*-infected leaves, it was reported that both tryptamine accumulation and DNA fragmentation are playing an important role in light-induced resistance of Sekiguchi lesion mutant (Ueno et al., 2004). In *B. oryzae*-infected leaves, tryptamine accumulation was also significantly induced in leaves with the Sekiguchi lesions under light, but not in those with brown spot lesions by dark and DCMU treatments (Fig. 5b). In a previous report (Ueno et al., 2005), we indicated that tryptamine inhibited the spore germination and growth of infection hyphae of *B. oryzae* at a concentration of over 150 μg/ml. These results suggested that tryptamine might be a key antifungal component for no sporulation of *B. oryzae* in the Sekiguchi lesion.

In *M. grisea* infection, DNA fragmentation was detected in the Sekiguchi lesions under light, but in blast lesions in the dark. Ueno et al. (2004) reported that DNA fragmentation was induced by H_2O_2 generated by tryptamine oxidation. In *B. oryzae* infection, DNA fragmentation was observed in both leaves with brown spot lesions in the dark and with the Sekiguchi lesions under light. However, DNA fragmentation was significantly induced in leaves with the Sekiguchi lesions under light, as compared with those in leaves with brown spot lesions in the dark. This result showed that DNA fragmentation in *B. oryzae*-infected leaves is light-dependently induced. It is reported that cell-death-inducing treatment by the fungal toxin or the plant activator significantly increased resistance to semi-biotrophic fungus *M. grisea*, while the same treatment was ineffective against necrotorophic fungus *B. oryzae* (Ahn et al., 2005). These results suggested that cell death induced by DNA fragmentation was effective to inhibition of hyphal growth of *M. grisea*, but ineffective to that of *B. oryzae*. DNA fragmentation and tryptamine accumulation were significantly inhibited in DCMU-pretreated leaves even under light. Ueno et al. (2008) reported that DNA fragmentation in Sekiguchi lesion mutant infected by *M. grisea* was induced by tryptamine-dependent H_2O_2.

Light-induced resistance of rice against *B. oryzae* is supported by tryptamine with antifungal activity, and increased DNA fragmentation might contribute to enhance the accumulation of tryptamine involved in inhibition of

pathogen development such as sporulation.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Young Scientists (B) (No. 21780037) from The Ministry of Education, Sports, Science and Technology of Japan.

Reference

Ahn, I.-P., Kim, S., Kang, S., Suh, S.-C. and Lee, Y.-H. (2005) Rice defense mechanisms against *Cochliobolus miyabeanus* and *Maganaporthe grisea* are distinct. Phytopathology, 95: 1248-1255.

Arase, S., Fukuyama, R., Tokizawa, K., Ikegami, S., Honda, Y. and Nozu, M. (1997) The effects of light and photo - and protein-synthetic inhibitors on the Sekiguchi lesion formation by *Magnaporthe grisea* in rice cv. Sekiguchi-asahi. Journal of Phytopathology, 145: 31-35.

Arase, S., Fukuyama, R. and Honda, Y. (2000a). Light-dependent induction of the Sekiguchi lesion formation by *Bipolaris oryzae* in rice cv. Sekiguchi-asahi. Journal of Phytopathology, 148: 193-196.

Arase, S., Fujita, K., Uehara, T., Honda, Y. and Isota, J. (2000 b) Light-enhanced resistance to *Magnaporthe grisea* infection in the rice Sekiguchi lesion mutant. Journal of Phytopathology, 148: 197-203.

Arase, S., Ueno, M., Toko, M., Honda, Y., Itoh, K. and Ozoe, Y. (2001) Light-dependent accumulation of tryptamine in the rice Sekiguchi lesion mutant infected with *Magnaporthe grisea*. Journal of Phytopathology, 149: 409-413.

Dangl, JF., Dietrich, RA. and Richberg, MH. (1996) Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell, 8: 1793-1807.

Ryals, JA., Neuenschwander, UH., Willits, MG., Molina, A., Steiner, HY. and Hunt, MD. (1996) Systemic acquired resistance. Plant Cell, 8: 1809-1819.

Tanaka, N., Nakajima, Y., Kaneda, T., Takayama, S., Che, FS. and Isogai, A. (2001) DNA laddering during hypersensitive cell death in cultured rice cell induced by an incompatible strain of *Pseudomonas avenae*. Plant Biotechnology, 18: 295-299.

Ueno, M., Kihara, J., Honda, Y. Isota, J. and Arase, S. (2004) DNA fragmentation in Sekiguchi lesion mutants of rice infected with *Magnaporthe grisea*. Journal of General Plant Pathololgy, 70: 321-328.

Ueno, M., Imaoka, A., Kihara, J. and Arase, S. (2005) Tryptamine accumulation in Sekiguchi lesion mutant of rice infected with *Bipolaris oryzae*. Bulletin of the Faculty of Life and Enviromental Science, Shimane University, 10: 9-12.

Ueno, M., Imaoka, A., Kihara, J. and Arase, S. (2007) Effect of light quality on induction of tryptamine-mediated resistance in lesion mimic mutant of rice infected with *Magnaporthe grisea*. Journal of Phytopathology, 155: 228-235.

Ueno, M., Imaoka, A., Kihara, J. and Arase, S. (2008) Tryptamine pathway-mediated DNA fragmentation is involved in Sekiguchi lesion formation for light-enhanced resistance in lesion mimic mutant of rice to *Maganaporthe grisea* infection. Journal of Phytopathology, 156: 715-724.

Imaoka, A., Ueno, M., Kihara, J. and Arase, S. (2008) Effect of photo-synthetic inhibitor on tryptamine pathway-mediated Sekiguchi lesion formation in lesion mimic mutant of rice infected with *Magnaporthe grisea*. Journal of Phytopathology, 156: 522-529.