Original Paper Plant Biotechnology, **21** (2), 113-118 (2004)

Expression of the AtRAD51 Gene Promoter in Response to DNA Damage in Transgenic Tobacco

Tomohide MAEDA[1], Yuriko WATAKABE[2], Seunghee SEO[1], Hisabumi TAKASE[2a] and Kazuyuki HIRATSUKA[1]*

[1]*Division of Plant Biotechnology, Graduate School of Environment and Information Sciences, Yokohama National University, Yokohama 240-8501, Japan*
[2]*Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0101, Japan*
[a]*Present address: Research Institute of Innovative Technology for the Earth (RITE), Kyoto 619-0292, Japan*
**Corresponding author* E-mail address: hiratsk@ynu.ac.jp

Received 10 November 2003; accepted 19 January 2004

Abstract

AtRAD51 gene encodes an *Arabidopsis* homologue of *E. coli* RecA protein that catalyzes homologous recombination and repair of chromosomal DNA. The *AtRAD51* mRNA expression is regulated by DNA damage and cell cycle, but the mechanisms and signal transduction pathway involved in the *AtRAD51* transcriptional regulation are largely unknown. In order to investigate regulatory mechanisms of DNA-damage induced gene expression in plants, we carried out functional analysis of the *AtRAD51* gene promoter. A 0.7 kb fragment of the 5′-upstream region of *AtRAD51* genomic DNA was fused to the firefly luciferase reporter gene and introduced into tobacco cells by microprojectile bombardment and *Agrobacterium*-mediated transformation. Induction experiment using bleomycin indicated that the *AtRAD51* promoter is able to direct gene expression in tobacco cells in response to DNA damage.

Key words: DNA damage, luciferase assay, promoter, *RAD51*.

Abbreviations

CAB, chlorophyll a/b-binding protein; CaMV, cauliflower mosaic virus; DLRA, dual-luciferase reporter assay; Fluc, firefly luciferase; GUS, β-glucuronidase; Rluc, *Renilla* luciferase; RT-PCR, reverse transcription-polymerase chain reaction.

Introduction

All living cells respond to genotoxic stresses by expressing a set of genes under the control of an inducible promoter that respond to signals generated by DNA damage. Such cellular responses are essential for normal cell cycle progression and maintenance of genome integrity. One example is the *RAD51* gene that shows structural similarity to a DNA recombination protein RecA from *E. coli* (Shinohara and Ogawa, 1999). In *Saccharomyces cerevisiae*, a series of mutants sensitive to DNA-damaging agents have been isolated and characterized (Game, 1993). Plants respond to various environmental stresses such as light, temperature, drought and pathogen attack by expressing genes under the control of inducible promoters that respond to particular regulatory signals. Because of being constantly exposed to DNA-damaging UV light irradiation, plants have developed protection mechanisms against DNA-damaging agents (Britt *et al.*, 1996; Jansen *et al.*, 1998). Plant cell responds to DNA damage in many aspects of physiological processes including expression of a set of genes that involved in DNA repair. Recent studies on *Arabidopsis* genes demonstrated that many genes are up-regulated by the treatment with DNA-damaging agents (Chen *et al.*, 2003). Although factors that control sensing and signal transduction of DNA damage have been identified in *Arabidopsis*, the regulatory mechanisms involved in gene expression in response to DNA damage are not clear (Garcia *et al.*, 2003). Moreover, the promoter sequences that direct gene expression in response to DNA damage have not been analyzed in plants.

Previous studies demonstrated the existence of multiple genes encoding RecA-like proteins in *Arabidopsis*. The *AtRAD51* gene was identified as a RecA-like gene that shows high similarity to the yeast *RAD51* gene and has shown to be induced by

DNA damage (Klimyuk and Jones, 1997; Doutriaux *et al.,* 1998; Osakabe *et al.,* 2002). In order to investigate the mechanisms involved in regulated expression of genes in response to DNA damage, we chose the *AtRAD51*, because of the availability of the expression profiles against DNA damaging agents and the genomic DNA sequence information (Urban *et al.,* 1996). Moreover, recent studies of genes responding to genotoxic stress demonstrated that the induction level of *AtRAD51* gene is one of the best among the up-regulated genes that respond to DNA damage (Chen *et al.,* 2003).

We conducted this study as a part of our efforts to uncover the mechanisms involved in the response to DNA damage in higher plants. As a first step towards understanding the specific regulation of gene expression by DNA damage, we analyzed the gene regulation in response to DNA damage. Using a transient assay and transgenic plants, we characterized the regulated expression of the *AtRAD51* gene and demonstrated that the *AtRAD51* promoter is able to direct gene expression in response to DNA damage.

Materials and methods

Construction of plasmids

The sequence of *AtRAD51* promoter region was amplified from the genomic DNA of *Arabidopsis thaliana* (Col-0) by PCR with primers; *AtRAD51*-U; 5′-GG**AAGCTT**CACCGGTTTGAACCCGGTTAG-3′ and *AtRAD51*-D; 5′-GG**CCATGG**TCGTCATTTCTCTCAATCAGAG-3′. The amplified fragment was digested with *Hin*dIII and *Nco*I and inserted into the *Hin*dIII-*Nco*I site of pBI221-luc+ (Matsuo *et al.,* 2001).

ArLIM15::Fluc was constructed by excising the *ArLIM15* promoter fragment from the plasmid pARLE3 that contains a 2.5 kb genomic DNA fragment of *ArLIM15* gene (Sato *et al.,* 1995) by digesting with *Hin*dIII and *Xba*I, blunted, and inserted into the *Hin*dIII-blunt site of pBI221-luc+. The pBI221*CAB1*::Fluc was constructed as described previously (Matsuo *et al.,* 2001). The binary vector constructs were made by joining *Hin*dIII-*Pvu*II digested promoter-Fluc fragments and the *Hin*dIII-*Eco*RI-blunt digested binary vector pBI121-LUC.

Microprojectile bombardment and transformation of plant cells

A transient expression assay by microprojectile bombardment was conducted as described previously (Matsuo *et al.,* 2001). Transformation and selection of tobacco and BY-2 cells using *Agrobacterium tumefacience* strain LBA4404 were carried out as described previously (Matsuoka and Nakamura, 1991).

Luciferase assay

Transient assay of luciferase reporter genes was conducted by the DLRA system as described previously (Matsuo *et al.,* 2001). Spatio-temporal detection of luciferase activity was carried out by the *in vivo* imaging system as described previously (Millar *et al.,* 1992; Watakabe *et al.,* 2001).

Results

Transient expression in tobacco BY-2 cells by microprojectile bombardment

To confirm the promoter activity directed by the DNA fragment inserted upstream of the luciferase reporter gene, we performed transient expression assay by microprojectile bombardment using tobacco BY-2 cells followed by DLRA. As a DNA damaging agent, we used a radiomimetic agent, bleomycin, because of its specificity for DNA damage (Menke *et al.,* 2001). The 5′-upstream region of *AtRAD51* amplified from the *Arabidopsis* genomic DNA was fused to the firefly luciferase gene and introduced into BY-2 cells together with the plasmid for internal reference, CaMV35S::Rluc. Promoter-Fluc fusion constructs containing promoter fragments from *ArLIM15* gene that encodes a meiosis-associated RecA-like protein (Sato *et al.,* 1995) and the light-inducible gene, *CAB1* (Matsuo *et al.,* 2001), were also tested for their response to bleomycin treatment (**Fig. 1**).

As shown in **Fig. 2**, *AtRAD51*::Fluc exhibited an elevated expression levels upon bleomycin treatment. Although significantly high expression levels were also observed without the treatment, induction of the promoter activity was detected under the experimental condition exploited in this study (Matsuo *et al.,* 2001). On the other hand, *CAB1*::Fluc construct showed a reduced level of expression upon bleomycin treatment. *ArLIM15* and CaMV35S promoter-Fluc constructs showed no significant changes in promoter activities by the identical treatment.

Induction of AtRAD51 promoter activity in transgenic tobacco BY-2 cells

We previously described an *in vivo* bioluminescence detection system using transgenic BY-2 cells harboring luciferase reporter gene for the detection of gene induction by treatment with chemicals (Watakabe *et al.,* 2001; Watakabe *et al.,* submitted).1 To characterize the promoter response

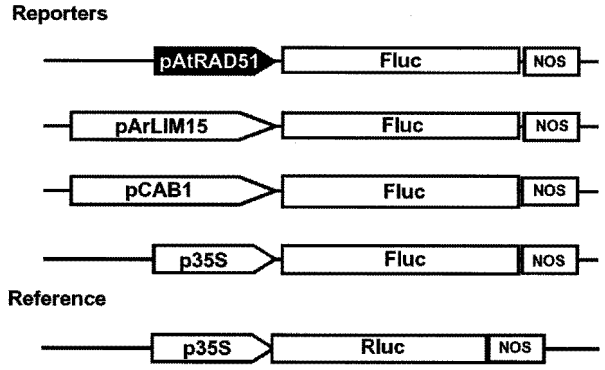

Fig. 1 Schematic representation of reporter and reference plasmid constructs used in this study.

pAtRAD51, a 0.7 kb fragment of the *AtRAD51* gene promoter; p35S, A 0.9 kb CaMV35S promoter sequence from the pBI221 vector; pCAB1, a 1.5 kb *CAB1* promoter sequence; pArLIM15, a 1.3 kb fragment of the *ArLIM15* gene promoter sequence; NOS, *nos*-terminator sequence from the pBI221 vector; Fluc, modified firefly luciferase gene; Rluc, *Renilla* luciferase gene.

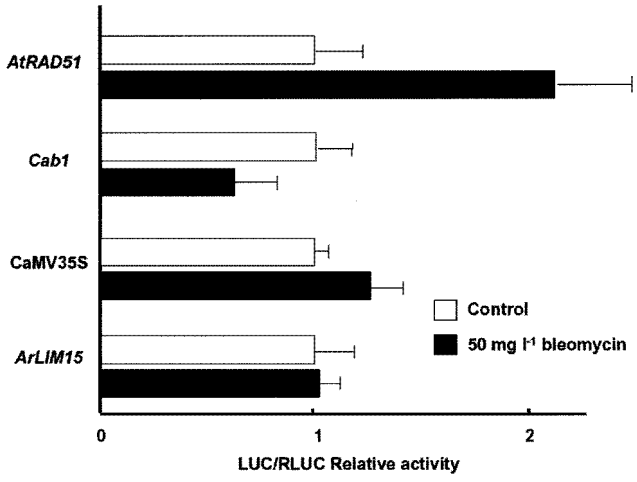

Fig. 2 Transient expression analysis of bleomycin treatment induced expression of various promoters in tobacco BY-2 cells.

Transient expression analysis of various promoters in tobacco BY-2 cells. Test plasmids were co-bombarded with an equal amount of reference plasmid followed by the bleomycin treatment (50 mg l^{-1} final conc.). DLRA was conducted 24 h post-bombardment. Relative activity represents the Fluc/Rluc ratio for each promoter normalized to the non-treatment sample. Values are the means of three independently bombarded samples with error bars representing standard deviation (SD).

with this system, we generated transgenic BY-2 cells by *Agrobacterium*-mediated transformation. As shown in **Fig. 3**, we could successfully obtain

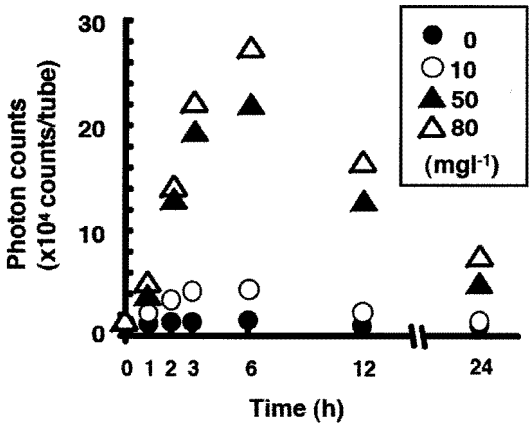

Fig. 3 Firefly luciferase activities in transgenic tobacco BY-2 cells harboring *AtRAD51*::Fluc.

Transformed BY-2 liquid culture cells (100 μl) were transferred to a plastic test tube and mixed gently with an equal volume of indicated concentrations of bleomycin aqueous solution containing 0.1 mM luciferin. Detection of luciferase activity at each time point was carried out using a luminometer.

expression profiles for *AtRAD51*::Fluc upon treatment at various concentrations of bleomycin. Fluc activity measurement demonstrated that the *AtRAD51* promoter was induced as early as 3 h after bleomycin treatment. The promoter activity reached its maximal level in 6 to 12 h after the induction treatment and gradually decreased over 24 to 48 h. Data obtained in this assay also demonstrated that the induction level of the *AtRAD51* promoter is dependent on the concentration of bleomycin (**Fig. 3**). Transgenic BY-2 cells harboring CaMV35S::Fluc did not show any induction of luciferase activity by bleomycin (data not shown).

Induction of AtRAD51 promoter activity in transgenic tobacco

To test the *AtRAD51* promoter inducibility in transgenic plants, we generated transgenic tobacco plants harboring *AtRAD51*::Fluc. In order to select transgenic lines with better *AtRAD51* promoter inducibility, we tested four randomly selected T2 transgenic tobacco lines for bleomycin treatment. Five-day-old transgenic tobacco seedlings were treated with 50 mg l^{-1} bleomycin aqueous solution in a test tube and the entire light emission was monitored in the luminometer. Induction of luciferase activity was observed in all four lines tested (data not shown).

The bleomycin treatment was conducted by applying a drop of bleomycin solution on the surface of a detached mature leaf. As shown in **Fig. 4**, strong induction of the *AtRAD51* promoter activity

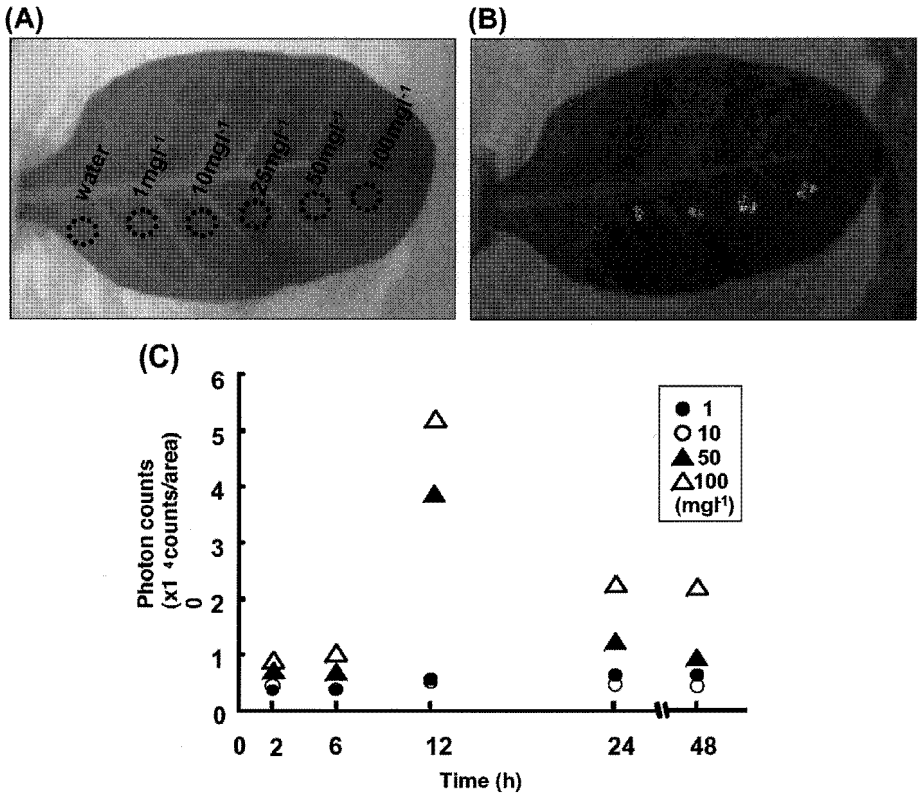

Fig. 4 Induction of *AtRAD51* promoter activity in transgenic tobacco leaf.

Light emission from *AtRAD51* promoter::Fluc transgenic tobacco leaf was monitored by low-light video image analysis following treatment with 2 μl of various concentrations of bleomycin in aqueous solution. (A) Bright field image. (B) Luciferase bioluminescence from the bleomycin treated leaf. (C) Time-course measurements of luciferase bioluminescence from different concentrations of bleomycin treated area by photon counting. Luminescence image from the leaf was taken at 12 h after bleomycin application. The sample was sprayed once with 0.1 mM luciferin and the image was obtained after 1 h of photon collection.

was detected as a discrete emission of light from the surface of the bleomycin treated leaf. No systemic induction of *AtRAD51* promoter activity was observed in this assay system; the expression of the *AtRAD51*::Fluc was confined to the bleomycin-treated area. The bleomycin-dependent induction of promoter activity was not observed with this treatment on transgenic tobacco plants harboring promoter-luciferase gene fusion of the salicylic acid inducible pathogenesis-related protein 1a gene (Watakabe *et al.*, 2001), *CAB1*::Fluc and *CaMV35S*::Fluc (data not shown).

Luciferase activity measurement revealed that the induction of the *AtRAD51* promoter activity by the bleomycin treatment was detectable within 3 h. The promoter activity reached its maximal level in 12 h after the induction treatment and gradually decreased over 24 to 48 h (**Fig. 4**).

Discussion

Recent studies on DNA damage-responsive genes in plants revealed the existence of a set of genes induced by genotoxic stress treatment with DNA damaging agents. Searches for cis-regulatory elements within the putative promoter region of several genotoxic stress responsive genes revealed some putative consensus sequences. However, no functional studies of the DNA damage responsive promoter have been conducted (Chen *et al.*, 2003).

To identify the DNA damage responsive promoter from higher plants, we tested transient expression of the *AtRAD51* promoter by microprojectile bombardment. Data obtained in this study suggest that the *AtRAD51* promoter is functional in tobacco BY-2 cells, induced upon bleomycin treatment, and therefore the promoter is DNA damage responsive. The *ArLIM15* gene encodes a RecA-like protein closely related to RAD51 protein and its expression has been shown to be meiosis-associated (Sato *et al.*,

1995; Klimyuk and Jones, 1997). In this study, *ArLIM15*::Fluc was not induced by the bleomycin treatment. This observation is consistent with the results of previous studies (Doutriaux *et al.*, 1998; Osakabe *et al.*, 2002). On the other hand, the expression level of *CAB1*::Fluc was down-regulated upon bleomycin treatment. Although molecular basis for this down-regulation of gene expression in response to DNA damage is unknown, these results suggest the existence of a complex signaling network in which bleomycin-induced cellular responses are involved.

A previous study using *Arabidopsis* tissue culture cells indicated that the *AtRAD51* expression is cell cycle dependent and induced during G1/S phase transition (Doutriaux *et al.*, 1998). On the other hand, the *AtRAD51*::Fluc expression levels in transgenic BY-2 cells obtained in this study were relatively low. These results suggest that the cell cycle-dependent regulatory mechanism of the *AtRAD51* promoter is not fully functional in transgenic tobacco cells. However, the induction by bleomycin treatment revealed that the *AtRAD51* promoter responds highly to DNA damage in transgenic BY-2 cells. This result indicates a possibility that distinct regulatory mechanisms; cell cycle-dependent and DNA damage-responsive pathways are involved in *AtRAD51* transcriptional regulation. The findings obtained on the *Arabidopsis ATM* gene, the gene that is mutated in the human disease ataxia-telangiectasia (AT), also support this possibility; the *atm* mutation resulted in reduced induction levels of genes responding DNA damage including *AtRAD51*, while their non-induced expression levels are unaffected (Garcia *et al.*, 2003).

Using tobacco mature leaves, we could observe discrete induction of *AtRAD51*::Fluc activity upon bleomycin treatment. Interestingly, the induced cells were restricted to the bleomycin-treated area. This suggests that the induction mechanism is cell-autonomous and no systemic induction system is involved in this regulated gene expression.

In this study, we tested only bleomycin as a DNA-damaging agent. Previous studies on the induction of *RAD51* gene expression have been done using gamma-ray irradiation treatment (Klimyuk and Jones, 1997; Doutriaux *et al.*, 1998). In the present study, the *AtRAD51* promoter activity was maximal about 6 to 12 h after bleomycin treatment, while the induction of the *AtRAD51* mRNA accumulation was reportedly maximal within 30 to 60 min after the gamma-ray irradiation (Garcia *et al.*, 2003). It is possible that the difference in the mode of DNA damage may result in different regulation patterns. Alternatively, the difference in temporal expression pattern may simply be explained by the difference between tobacco and *Arabidopsis* system. Further studies are necessary to highlight the similarity and differences of these two systems. In addition, we should be careful when comparing the mode of induction of DNA damage in response to different genotoxic treatments.

Previous studies on reporter gene technology for monitoring DNA damage or genotoxic stress mainly depended on a homologous recombination system based on the reporter gene reconstitution (Swoboda *et al.*, 1994; Puchta *et al.*, 1995; Kovalchuk *et al.*, 2000). Only one example of functional promoter-GUS fusion of a DNA-damage-responsive gene in higher plants has been described so far (Deveaux *et al.*, 2000). To our knowledge, this is the first report on real-time monitoring system of the responses to DNA damage in higher plants. Although further studies are necessary, this DNA-damage reporter system will be useful for investigating the regulatory mechanisms and signaling pathways in which genotoxic stress-inducible cellular responses are involved. Moreover, by further improving the promoter responsiveness and specificity against genotoxic substances, we hope to develop a novel sensing system for the detection and monitoring the environmental DNA damaging agents.

Acknowledgments

This work was supported by the Japanese Society for the Promotion of Science Grant-in-Aid for Scientific Research (No. 14360205) and by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 12052217). T.M. is supported by the 21st Century COE Research Program "Bio-Eco Environmental Risk Management" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Britt, A. B., 1996. DNA damage and repair in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol., **47**: 75-100.

Chen, I.-P., Haehnel, U., Altschmied, L., Schubert, I., Puchta, H., 2003. The transcriptional response of *Arabidopsis* to genotoxic stress - a high-density colony array study (HDCA). Plant J., **35**: 771-786.

Deveaux, Y., Alonso, B., Pierrugues, O., Godon, C., Kazmaier, M., 2000. Molecular cloning and developmental expression of AtGR1, a new growth-related *Arabidopsis* gene strongly induced by ionizing radiation. Radiat. Res., **154**: 355-364.

Doutriaux, M. P., Couteau, F., Bergounioux, C., White, C., 1998. Isolation and characterisation of the *RAD51* and *DMC1* homologs from *Arabidopsis thaliana*. Mol. Gen. Genet., **257**: 283-291.

Game, J. C., 1993. DNA double-strand breaks and the *RAD50-RAD57* genes in *Saccharomyces*. Semin. Cancer Biol., **4**: 73-83.

Garcia, V., Bruchet, H., Camescasse, D., Granier, F., Bouchez, D., Tissier, A., 2003. AtATM is essential for meiosis and the somatic response to DNA damage in plants. Plant Cell, **15**: 119-132.

Jansen, M. A. K., Gaba, V., Greenberg, B. M., 1998. Higher plants and UV-B radiation: Balancing damage, repair and acclimation. Trends Plant Sci., **3**: 131-135.

Klimyuk, V. I., Jones, J. D. G., 1997. AtDMC1, the *Arabidopsis* homologue of the yeast DMC1 gene, characterization, transposon-induced allelic variation and meiosis-associated expression. Plant J., **11**: 1-14.

Kovalchuk, O., Arkhipov, A., Barylyak, I., Karachov, I., Titov, V., Hohn, B., Kovalchuk, I., 2000. Plants experiencing chronic internal exposure to ionizing radiation exhibit higher frequency of homologous recombination than acutely irradiated plants. Mutat. Res., **449**: 47-56.

Matsuo, N., Minami, M., Maeda, T., Hiratsuka, K., 2001. Dual luciferase assay for monitoring transient gene expression in higher plants. Plant Biotechnol., **18**: 71-75.

Matsuoka, K., Nakamura, K., 1991. Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc. Natl. Acad. Sci. U. S. A., **88**: 834-838.

Menke, M., Chen, I.-P., Angelis, K. J., Schubert, I., 2001. DNA damage and repair in *Arabidopsis thaliana* as measured by the comet assay after treatment with different classes of genotoxins. Mutat. Res., **493**: 87-93.

Millar, A. J., Short, S. R., Hiratsuka, K., Chua, N.-H., Kay, S. A., 1992. Firefly luciferase as a reporter of regulated gene expression in higher plants. Plant Mol. Biol. Rep., **10**: 324-337.

Osakabe, K., Yoshioka, T., Ichikawa, H., Toki, S., 2002. Molecular cloning and characterization of RAD51-like genes from *Arabidopsis thaliana*. Plant Mol. Biol., **50**: 71-81.

Puchta, H., Swoboda, P., Hohn, B., 1995. Induction of homologous DNA recombination in whole plants. Plant J., **7**: 203-210.

Sato, S., Hotta, Y., Tabata, S., 1995. Structural analysis of a recA-like gene in the genome of *Arabidopsis thaliana*. DNA Res., **2**: 89-93.

Shinohara, A., Ogawa, T., 1999. RAD51/RecA protein families and the associated proteins in eukaryotes. Mutat. Res., **435**: 13-21.

Swoboda, P., Gal, S., Hohn, B., Puchta, H., 1994. Intrachromosomal homologous recombination in whole plants. EMBO J., **13**: 484-489.

Urban, C., Smith, K. N., Beier, H., 1996. Nucleotide sequences of nuclear tRNA(Cys) genes from *Nicotiana* and *Arabidopsis* and expression in HeLa cell extract. Plant Mol. Biol., **32**: 549-552.

Watakabe, Y., Ono, S., Hiratsuka, K., 2001. Characterization of agents that induce acquired resistance by transgenic plants. J. Pesticide Sci., **26**: 296-299 (in Japanese).