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Original Paper

Non-destructive bioluminescence detection system for monitoring defense gene expression in tobacco BY-2 cells

Yuriko Watakabe[1], Sachiko Ono[2], Tsuneyuki Tanaka[2], Kazuyuki Hiratsuka[2,*]

[1] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan;
[2] Graduate School of Environment and Information Sciences, Yokohama National University, Hodogaya, Yokohama, Kanagawa 240-8501, Japan
* E-mail: hiratsk@ynu.ac.jp Tel & Fax: +81-45-339-4413

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Abstract Using tobacco BY-2 cells transformed with a promoter-luciferase gene fusion, we developed a non-destructive and sensitive *in vivo* assay system for monitoring defense gene expression in higher plant cells. A promoter fragment of a tobacco salicylic acid (SA) inducible pathogenesis-related gene, *PR-1a* isolated from the genomic DNA of tobacco BY-2 cells, was fused to the luciferase reporter gene and introduced into plant cells by Agrobacterium-mediated transformation. To detect the *PR-1a* promoter expression as a luciferase activity, transformed cells were mixed with luciferin solution and the bioluminescence levels were monitored *in vivo* using a conventional luminometer. Because the *PR-1a* promoter expression levels of the BY-2 cells are relatively high, the induction of the luciferase activities by the treatment with SA was barely detectable under log phase growth conditions. However, we could observe concentration dependent induction of the luciferase activities following SA application under stationary phase. Treatment with benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) and methyl-2,6-dichloroisonicotinic acid (INA) also resulted in a drastic increase in luciferase activities of transgenic cells in a dose dependent manner. On the other hand, treatment with an inactive SA analog 4-hydroxybenzoic acid (4-HBA) showed no influence on luciferase activities. The sensitivity of the assay system was higher than the previously reported techniques for the detection of induction by SA or BTH. These results indicate that this rapid, inexpensive and versatile assay system would be useful for the identification and characterization of chemicals capable of inducing defense gene expression in higher plant cells.

Key words: *Agrobacterium tumefaciens*, bioluminescence, BY-2, defense gene expression, firefly luciferase, *Nicotiana tabacum*, salicylic acid.

Quantification of defense gene expression is an excellent way of monitoring the induction of defense responses in plants. Pathogen infection or the treatment with chemicals capable of inducing defense responses in plants such as salicylic acid (SA) leads to the strong induction of transcription activation of defense related genes followed by the accumulation of mRNAs and proteins. Quantification of protein or mRNA accumulation levels of pathogenesis-related (PR) gene can be used for monitoring defense gene expression (Buchel and Linthorst 1999), but is a laborious and time-consuming process.

By fusing the reporter gene to a genomic DNA fragment, and examining the expression of the reporter gene under the control of the promoter, we can monitor the promoter activity indirectly as the enzymatic activity of the reporter gene product. We can rapidly detect and quantify the promoter activity using transgenic plant cells harboring the promoter-reporter gene. In higher plants, the GUS gene is the most popular reporter gene used for the analysis of tissue and organ-specific expression. The firefly luciferase (LUC) also is widely used as the reporter because the assay system enables us to conduct a non-invasive *in vivo* bioluminescence assay for monitoring gene expression in higher plants (Ow et al. 1986; Millar et al. 1992). In an attempt to develop a sensitive and versatile assay system for monitoring defense gene expression we have investigated the use of the promoter-LUC gene fusion for the study of regulated expression of defense genes in plant cells (Ono et al. 2004; Tanaka et al. 2006; Watakabe et al. 2001). Here we describe a novel assay protocol based on the LUC bioluminescence reporter system capable of detecting *PR-1a* gene induction in tobacco BY-2 cells. The system

Abbreviations: BTH, benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester; CaMV, *Caulflower mosaic virus*; 4-HBA, 4-hydroxybenzoic acid; GUS, β-glucuronidase; INA, methyl-2,6-dichloroisonicotinic acid; LUC, firefly luciferase; PCR, polymerase chain reaction; PR gene, pathogenesis-related gene; SA, salicylic acid

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enables us to conduct a highly sensitive, non-invasive and quantitative monitoring of activation of the *PR-1a* promoter by chemicals that induce defense gene expression.

Materials and methods

Isolation of PR-1a gene promoter fragment from tobacco BY-2 cell

Genomic DNA sample was isolated from the BY-2 cells cultured under the condition described previously (Nagata et al. 1992). Cells were harvested by centrifugation and crushed under liquid nitrogen using mortar and pestle. Extraction and purification of tobacco genomic DNA was carried out according to published methods (Murray and Thompson 1980). PCR amplification of *PR-1a* gene was conducted using the following primers containing a restriction endonuclease recognition sites (underlined);

PRF: 5′-GGGAAGCTTAAGGACTAAGATATACGAGG-3′

PRF3: 5′-GGGTCATGACTATAGGAGAAATGTTGTAT-3′.

The PCR was conducted by KOD DNA polymerase (Toyobo) under the condition recommended by the manufacturer. The reaction mixture was kept at 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, 58°C for 1 min, 74°C for 1min and then at 74°C for 3 min. The amplified 1.6 kb DNA fragment, separated by agarose gel electrophoresis, was digested by *Bsp*HI and filled-in by a DNA blunting kit (Takara). The DNA was then digested by *Hin*dIII and cloned into a *Hin*dIII- *Eco*RV site of pBluescript SKII+ (Stratagene). DNA sequence was carried out using a cycle sequencing with a Big-dye terminator kit (Perkin Elmer). The nucleotide sequence data of BY-2 *PR-1a* gene promoter region are available in DDBJ, EMBL and GenBank nucleotide sequence databases as accession number AB086949 (Figure 1A).

Plasmid construction

A genomic DNA fragment containing the *PR-1a* promoter was excised from the plasmid by *Hin*dIII/*Bsp*HI and inserted into pBI221-luc+ by replacing the CaMV35S promoter region by digesting with *Hin*dIII and *Nco*I (Matsuo et al. 2001). Then the plasmid was digested by *Pvu*II/*Hin*dIII and inserted into *Eco*RI-blunt/*Hin*dIII digested pBI121 (Clontech). To make the CaMV35S-luciferase construct, we excised the modified luciferase coding sequence from pSP-luc+ (Promega) by digesting *Bgl*II and *Eco*RV, then inserted it into *Sac*I-blunt/*Bam*HI digested pBI121 (Figure 1B). The plasmid was transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation using Genepulser (BioRad) according to the manufacturer's instructions.

Transformation by Agrobacterium

Tobacco BY-2 cells were transformed essentially as described previously (An 1987). Cells were used after 3 days after splitting the BY-2 cell culture. 4 µl acetosyringone (20 mM) was added to a 9 cm petri dish containing 4 ml of BY-2 cell. Using a 10 ml pipette, the cells were pipetted in and out about 30 times to induce lesions in the cells. 100 µl of over night culture of the bacterial cells were added to a petri dish and

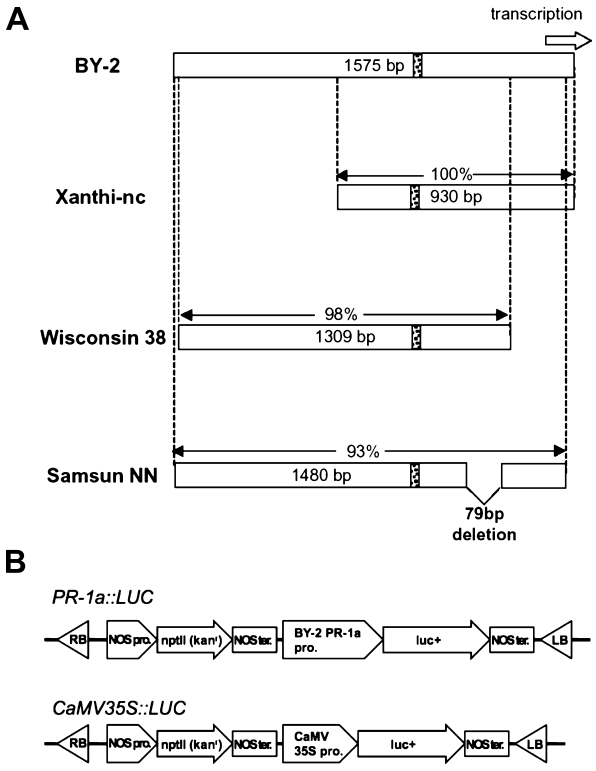

Figure 1. Sequence comparison of the *PR-1a* promoter region from different tobacco varieties (A) and the schematic structure of transgene cassettes used in this study (B). The degree of sequence homology between BY-2 *PR-1a* promoter sequence and previously described sequences are shown. Location of the *as-1*-like elements is indicated by stippled box. Accession numbers for BY-2 (this study), Xanthi-nc (Payne et al. 1988), Wisconsin38 (Gruner and Pfitzner 1994) and Samsun NN (Cornelissen et al. 1987) are AB086949, X12737, X76982 and X05959, respectively

RB and LB, T-DNA right and left borders, respectively; NOS pro., nopaline synthase gene promoter; nptII, neomycin phosphotransferase gene; NOS-ter., polyadenylation signal from the nopaline synthase gene; BY-2 PR-1a pro., promoter of tobacco *PR-1a* gene from BY-2; CaMV35S pro., promoter of cauliflower mosaic virus 35S RNA; luc+, modified firefly luciferase gene.

mixed thoroughly and then incubated for 3 days at 28°C. BY-2 cells were washed twice with liquid medium containing 500 mg l^{-1} carbenicillin and then plated on solid medium containing kanamycin (100 mg l^{-1}) and carbenicillin (500 mg l^{-1}). After the selection on solid medium for 3 weeks at 28°C, cells were transferred to the fresh solid medium for further selection. Isolated colonies were transferred to liquid medium containing kanamycin (100 mg l^{-1}) and carbenicillin (500 mg l^{-1}) for the establishment of cell lines. Cell lines were subcultured at one-week intervals.

in vivo Luciferase assay

Transformed BY-2 liquid culture cells (100 µl) were transferred to a plastic test tube (Sarstedt 55.476 PS) with using a Cell-Savor tip (Bio-Bik) and mixed gently with an equal volume of 1 mM luciferin (D-luciferin potasium salt, Molecular Probe) aqueous solution. For the promoter induction study, luciferin was added 24 h before the treatment. For treatments with chemicals, cells were adjusted to the desired concentration by

adding the concentrated stock solution containing 10% dimethylsulfoxide (DMSO). Final concentration of DMSO was adjusted to 1% for each treatment and the control. Detection of luciferase activity was carried out using a Lumat LB9501 luminometer (Berthold). During the intervals, cells were maintained in the dark at 28°C in the test tube with occasional shaking.

Results

Because of its clear responsiveness to SA treatment in tobacco BY-2 cells (Horvath and Chua 1996), we chose a tobacco *PR-1a* gene promoter for the construction of the luciferase fusion gene. A DNA fragment containing the *PR-1a* promoter region was amplified from the tobacco BY-2 genomic DNA by PCR primers designed based on the sequence information described previously (Cornelissen et al. 1987). As shown in Figure 1A, the *PR-1a* promoter sequence obtained from the BY-2 genomic DNA revealed an almost identical DNA sequence within the TATA-proximal region of the previously reported tobacco *PR-1a* gene sequences deposited in the databases. The *as-1*-like element that has shown to be responsible for induction of the *PR-1a* promoter is conserved, however, several sequence substitutions and insertions were observed within the upstream region of the promoter. The BY-2 genomic *PR-1a* fragment, containing a possible 5′ non-coding region with the first methionine codon, was fused in-frame to the LUC coding sequence.

Introduction of *PR-1a::LUC* gene fusion into tobacco BY-2 cells by high efficiency transformation by *Agrobacterium* resulted in a large number of transformed cell lines. In order to establish transgenic cell lines suitable for *in vivo* bioluminescence detection of regulated expression of *PR-1a* promoter, we selected transgenic cell lines that show normal growth. Next, we conducted a series of luciferase assays for the selection and evaluation of cell lines capable of detecting SA-dependent induction of luciferase activity. Seven-day-old randomly selected BY-2 transgenic cell lines treated with 300 µM SA showed various expression levels, but most of them exhibited a marked increase in luciferase activities upon SA treatment. On the other hand, similar treatment with BY-2 cells containing luciferase reporter gene under the control of the cauliflower mosaic virus 35S promoter did not show any induction of luciferase activity by SA treatment (data not shown).

In order to examine the influence of culture conditions on this assay system, we compared SA-dependent induction levels of luciferase activities under different culture conditions. Although the induction of luciferase activity was evident in cells at the stationary phase (Figure 2B, D), considerably high background levels of luciferase activity were detected from cells at the logarithmic phase (Figure 2A, C). On the other hand, a gradual decrease of the luciferase expression levels was observed when BY-2 cells harboring the *CaMV35S::LUC* construct were tested under the same condition (Figure 2E-H). The time-course of *PR-1a::LUC* activity obtained by *in vivo* bioluminescence assay was very similar to the previously described *PR-1a* mRNA accumulation pattern in BY-2 cells obtained by RNA gel blot analysis (Horvath and Chua 1996). Induction of the *PR-1a::LUC* was detectable as early as 6 h after SA treatment, and reached its maximal level in 24 to 36 h.

We examined the induction of *PR-1a::LUC* by treatment with BTH and INA at various concentrations in comparison with that by treatment with SA. Time-course measurements of *in vivo* bioluminescence assay after the addition of BTH, INA and SA resulted in the concentration-dependent induction of *PR-1a::LUC* activities. However, each compound exhibited a unique induction pattern. *PR-1a::LUC* expression levels obtained by BTH treatment were comparable to those obtained by SA treatment, but the expression timing was delayed. On the other hand, the *PR-1a::LUC* expression induced by INA treatment was slow and weak under the experimental condition used in this study. Treatment with 4-hydroxybenzoic acid (4-HBA) resulted in no detectable increase of luciferase activity at any concentration (Figure 3). Maximal induction levels were observed at higher concentrations, but treatment with 500 µM BTH lowered the activities of *PR-1a::LUC* after 36 to 48 h incubation. Constant reduction of luciferase activities similar to SA treatment were observed in transgenic BY-2 cells harboring *CaMV35S::LUC* (data not shown).

Discussion

For the development of an assay system suitable for monitoring the expression of defense genes we exploited the tobacco *PR-1a* gene as a model system because its regulated gene expression is tightly controlled by pathogen attack or by chemical treatment that induces SAR responses. Comparison of the *PR-1a* promoter nucleotide sequence obtained in this study with the previously described data deposited in the databases revealed a sequence polymorphism of *PR-1a* gene among *Nicotiana tabacum* cultivars. However, sequence diversity is rather confined to the upstream region relative to the transcription start site. Together with the results obtained in this study, this observation is consistent with the fact that the SA-dependent regulated expression of *PR-1a* promoter is predominantly determined by the TATA-proximal region of the promoter sequence (Hagiwara et al. 1993; Ohshima et al. 1990).

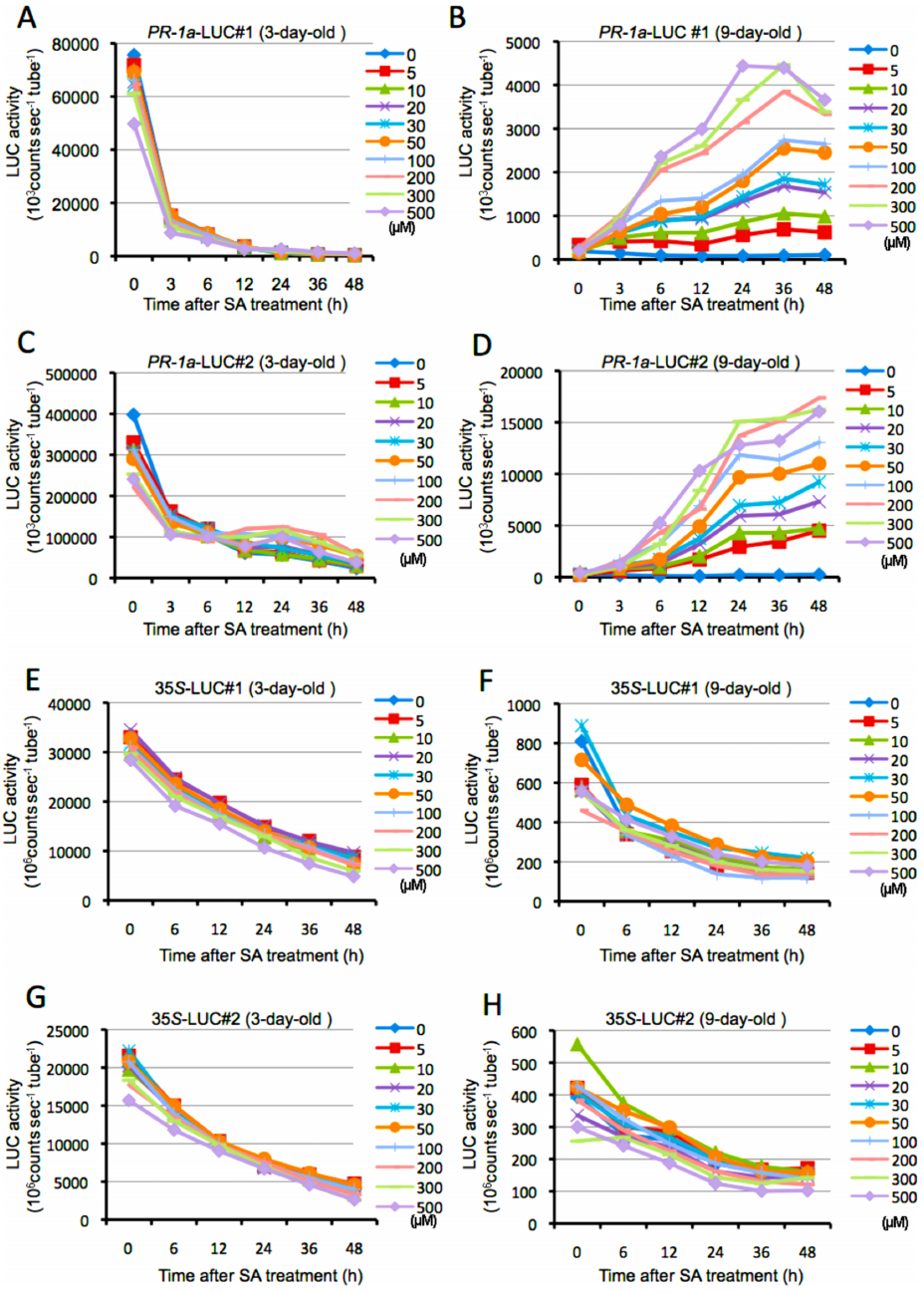

Figure 2. Time-course measurement of firefly luciferase activities in intact transgenic tobacco BY-2 cells harboring *PR-1a::LUC* and *CaMV35S::LUC* under different culture conditions and various SA concentrations. (A) *PR-1a*-LUC cell line #1 at logarithmic phase (3-day-old culture). (B) *PR-1a*-LUC cell line #1 at stationary phase (9-day-old culture). (C) *PR-1a*-LUC cell line #2 at logarithmic phase (3-day-old culture). (D) *PR-1a*-LUC cell line #2 at stationary phase (9-day-old culture). (E) CaMV35S-LUC cell line #1 at logarithmic phase (3-day-old culture). (F) CaMV35S-LUC cell line #1 at stationary phase (9-day-old culture). (G) CaMV35S-LUC cell line #2 at logarithmic phase (3-day-old culture). (H) CaMV35S-LUC cell line #2 at stationary phase (9-day-old culture). Similar bioluminescence expression patterns were observed from two independently established transformed cell lines (#1 and #2).

The firefly luciferase has been used as a reporter for the *in vivo* monitoring of regulated gene expression in plants. Previous studies on the promoter characterization and mutant isolation with promoter-luciferase fusion genes have been carried out successfully. However, a problem associated with the *in vivo* luciferase assay for monitoring *PR* gene expression has been reported. In fact, tomato *PR* gene promoters were shown to be induced by luciferin treatment itself in *Arabidopsis* (Jorda and Vera 2000). Moreover, because the bioluminescence reaction of firefly luciferase consumes ATP and oxygen, it is possible that a high level expression of luciferase in the presence of luciferin may interfere with a normal cellular response. In this study, however, we did not observe any induction of the *PR-1a* promoter activities with luciferin application. The *PR-1a*

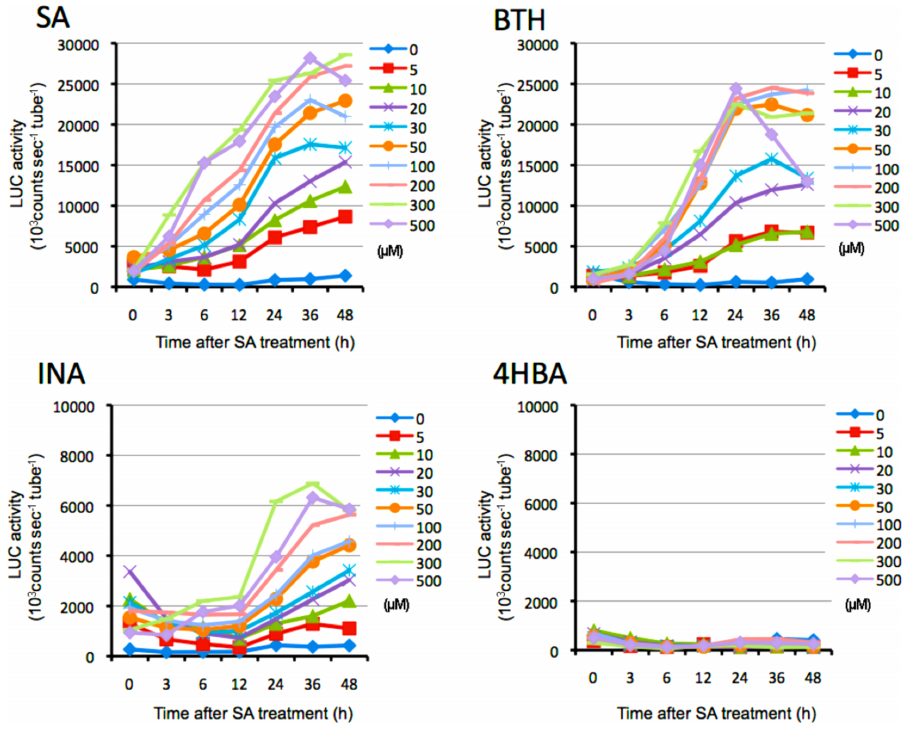

Figure 3. Time-course measurement of firefly luciferase activities in intact transgenic tobacco BY-2 cells (cell line #1) harboring *PR-1a::LUC* following treatments of various concentrations of chemicals capable of inducing defense gene expression. (A) SA. (B) BTH. (C) INA. (D) 4HBA. Results are representative of triplicate experiments with similar results.

promoter activity was induced with SA, BTH and INA. On the other hand, treatment with an inactive SA analogue, 4-HBA, or blank control did not show increase in luciferase activities in the presence of luciferin (Figure 3). These results suggest that the induction of luciferase activity observed in our assay system is dependent upon chemicals capable of inducing defense genes. The difference in response to luciferin between the two systems may simply be explained by the difference in physiological condition i.e., mature plants and BY-2 cells. No influence on cellular responses by luciferase overexpression was observed under the condition used in this study even in the transgenic cells harboring *CaMV35S::LUC*. This suggests that the consumption of ATP and oxygen by luciferase is negligible in the present assay system.

Interestingly, our observation suggested that the *PR-1a::LUC* expression levels are affected by the tissue culture condition in BY-2 cells. As shown in Figure 2, a clear induction pattern of *PR-1a::LUC* can be observed in cells at the stationary phase. On the other hand, considerably high background expression levels of *PR-1a::LUC* were detected from cells at the logarithmic phase. Since no cell cycle dependent expression of tobacco *PR-1a* gene has been reported, this phenomenon might be specific to the *PR-1a::LUC* gene expression in BY-2 cells.

Previous study indicated that the CaMV35S promoter contains an *as-1* element that mediates immediate early SA response, however, no SA dependent induction of *CaMV35S::LUC* was detected under the assay condition of this study. This inconsistency may be due to the difference in promoter length; the CaMV35S promoter used in this study is derived from a 870 bp fragment of pBI221, on the other hand, relatively short promoter region (−343 to +8) was used in the previous study (Qin et al. 1994). Also, the difference in CaMV35S promoter responsiveness could be explained by the difference in cell type; tobacco leaves and BY-2 cells.

We used three representative *PR-1a* gene inducers, SA, BTH and INA for the evaluation of our assay system. Previous studies suggested that BTH and INA activate SAR signal transduction downstream or at the same site of SA (Conrath et al. 1995; Friedrich et al. 1996). Interestingly, the results shown in Figure 3 demonstrate that these chemicals showed distinct *PR-1a::LUC* expression patterns. SA turned out to be the most active compound as a *PR-1a::LUC* inducer in this particular assay system. The *PR-1a::LUC* induction levels obtained by BTH treatment were very similar to those obtained by SA treatment, but the temporal expression pattern was different. It would be interesting to investigate the relationship between the difference in *PR-1a::LUC* induction pattern and the mode of action of BTH and SA. Although INA is considered to be an active compound capable of inducing defense genes, the results obtained in this study suggest that the mode of induction of the *PR-1a* gene promoter by INA is distinct

from that by SA or BTH. Comparative studies for elucidating the differences in mode of induction by these compounds on transcription activation of defense related genes are currently underway.

In this report, we show that the transgenic BY-2 harboring *PR-1a::LUC* can be a powerful tool for studying the regulated expression mechanisms of defense genes of which activation is induced by factors such as SA and other chemicals capable of inducing SAR. In addition, the use of a BY-2 cell system would enable us to conduct an assay protocol with multi-well plates for the large-scale experiments (Albert et al. 2006; Narusaka et al. 2006). On the other hand, unlike the previously reported methods using a sophisticated detection system, the protocol described in this study only requires a conventional luminometer, and may also be suitable to small-scale experiments.

Using Arabidopsis *PR-1* gene promoter-luciferase gene fusion, two examples of isolation of mutants that exhibit altered *PR-1* gene expression have been reported (Maleck et al. 2002; Murray et al. 2002). However, quantitative data for *PR-1* promoter induction upon chemical treatment have not been reported in detail. On the other hand, we could conduct detailed quantitative monitoring of the defense gene induction in this study. As shown in Figure 3, induction by less than 5 μM of SA or BTH treatment was readily detectable in this assay system. Compared with the previously described induction experiments in response to treatment with SA or BTH, the sensitivity of this assay system was about 10 to 100 times higher (Lawton et al. 1996; Tanaka et al. 2006).

Using the present assay system, it would be possible to quantitatively monitor the activities of unknown chemicals capable of inducing defense gene expression (Nakashita et al. 2002). This quantitative gene expression monitoring system may be particularly useful for the high-throughput screening in the search for chemicals that induce defense responses in higher plant cells and the characterization of their properties.

Acknowledgements

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References

Albert P, Miya A, Hiratsuka K, Kawakami, N, Shibuya N (2006) A high-throughput evaluation system for *Arabidopsis* mutants for defense signaling. *Plant Biotechnol* 23: 459–466

An G (1987) Binary Ti vectors for plant transformation and Promoter analysis. *Meth Enzymol* 153: 293–305

Buchel AS and Linthorst HJM (1999) PR-1A group of plant proteins induced upon pathogen infection. In: Datta SK, Muthukrishnan S (eds) *Pathogenesis-related proteins in plants*. CRC Press, Boca Raton, pp 21–47

Conrath U, Chen Z, Ricigliano JR, Klessig DF (1995) Two inducers of plant defense responses, 2,6-dichloroisonicotinic acid and salicylic acid, inhibit catalase activity in tobacco. *Proc Natl Acad Sc USA* 92: 7143–7147

Cornelissen BJ, Horowitz J, van Kan JA, Goldberg RB, Bol JF (1987) Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. *Nucleic Acids Res* 15: 6799–6811

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Rella MG, Meier B, Dincher S, Staub T, Uknes S, et al. (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J* 10: 61–70

Gruner R, Pfitzner UM (1994) The upstream region of the gene for the pathogenesis-related protein 1a from tobacco responds to environmental as well as to developmental signals in transgenic plants. *Eur J Biochem* 220: 247–255

Hagiwara H, Matsuoka M, Ohshima M, Watanabe M, Hosokawa D, Ohashi Y (1993) Sequence-specific binding of protein factors to two independent promoter regions of the acidic tobacco pathogenesis-related-1 protein gene (PR-1). *Mol Gen Genet* 240: 197–205

Horvath DM, Chua N-H (1996) Identification of an immediate-early salicylic acid-inducible tobacco gene and characterization of induction by other compounds. *Plant Mol Biol* 31: 1061–1072

Jorda L, Vera P (2000) Local and systemic induction of two defense-related subtilisin-like protease promoters in transgenic Arabidopsis plants. Luciferin induction of *PR* gene expression. *Plant Physiol* 124: 1049–1058

Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant J* 10: 71–82

Maleck K, Neuenschwander U, Cade RM, Dietrch RA, Dangl JL, Ryals JA (2002) Isolation and characterization of broad-spectrum disease-resistant Arabidopsis mutants. *Genetics* 160: 1661–1671

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

Millar AJ, Short SR, Hiratsuka K, Chua N-H, Kay SA (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants. *Plant Mol Biol Reptr* 10: 324–337

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8: 4321–4325

Murray SL, Thomson C, Chini A, Read, ND, Loake GJ (2002) Characterization of a novel, defense-related Arabidopsis mutant, cir1, isolated by luciferase imaging. *Mol Plant Microbe Interact* 15: 557–566

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 Cell Line as the "HeLa" Cell in the Cell Biology of Higher Plants. *Int Rev Cytol* 132: 1–30

Nakashita H, Yoshioka K, Yasuda M, Nitta T, Arai Y, Yoshida S, Yamaguchi I (2002) Probenazole induces systemic acquired resistance in tobacco through salicylic acid accumulation. *Physiol Mol Plant Pathol* 61: 197–203

Narusaka M, Abe H, Kobayashi M, Kubo Y, Kawai K, Izawa N, Narusaka Y (2006) A model system to screen for candidate plant activators using an immune-induction system in *Arabidopsis*. *Plant Biotechnol* 23: 321–327

Ohshima M, Itoh H, Matsuoka M, Murakami T, Ohashi Y (1990) Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein gene in transgenic tobacco. *Plant Cell* 2: 95–106

Ono S, Tanaka T, Watakabe Y, Hiratsuka K (2004) Transient assay system for the analysis of *PR-1a* gene promoter in BY-2 cells. *Biosci Biotechnol Biochem* 68: 803–807

Ow DW, Wood KV, DeLuca M, de Wet JR, Helinski DR, Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* 234: 856–859

Payne G, Parks TD, Burkhart W, Dincher S, Ahl P, Metraux JP, Ryals J (1988) Isolation of the genomic clone for pathogenesis-related protein 1a from *Nicotiana tabacum* cv. Xanthi-nc. *Plant Mol Biol* 11: 89–94

Qin XF, Holuigue L, Horvath DM, Chua NH (1994) Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus as-1 element. *Plant Cell* 6: 863–874

Tanaka T, Ono S, Watakabe Y, Hiratsuka K (2006) Bioluminescence reporter assay system to monitor Arabidopsis *MPK3* gene expression in response to infection by *Botrytis cinerea*. *J Gen Plant Pathol* 72: 1–5

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)