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Development of a Screening System for Plant Defense-Inducing Agent using Transgenic Tobacco Plant with PR-1a Promoter and GUS Gene

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Pathogenesis-related protein-1a (PR-1a) is strongly induced in tobacco plants by pathogen attack, exogenous salicylic acid (SA) application and by other developmental processes. In order to develop a rapid screening system for the selection of plant defense-inducing compounds originated from various sources, we have transformed tobacco Samsun NN plants with a chimeric construct consisting of *GUS* (*β-glucuronidase*). In the T_1 generation, three transgenic lines having stable GUS expression were selected for further promoter analysis. Using GUS histochemical assay, we observed strong GUS induction driven by *PR-1a* promoter in *PR1a-GUS* transgenic tobacco leaves in response to the exogenous application of SA or benzol (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), a SA-derivative compound. In addition, *GUS* expression was maintained locally or systemically in *PR1a-GUS* transgenic line # 5 (T_2 generation) until after 3 days when they were treated with same chemicals. Our results suggested that the *PR1a-GUS* reporter gene system in tobacco plants may be applicable for the large-scale screening of defense-inducing substances.

Keywords : chemical screening, pathogenesis-related protein-1a, PR1a promoter-GUS transgenic tobacco plants, salicylic acid

In plants, pathogen-induced defense response is generally represented by the development of a hypersensitive response (HR). HR is defined by rapid, localized cell death at the pathogen infected site and is associated with restriction of the invaded pathogens as well as the activation of pathogenesis related (PR) proteins (Dangl et al., 1996). This response triggers a general resistance mechanism rendering the establishment of systemic acquired resistance (SAR) in the uninfected parts of the locally infected plant (Ryals et al., 1996).

In tobacco, several families of PR proteins have been described (Van Loon and Van Strien, 1999). Among them, PR-2 (glucanase) and PR-3 (chitinase) proteins have been shown to have antifungal properties. However, the biological functions of other PR proteins are not clear, yet (Van Loon and Van Strien, 1999). Although their functions are not clearly defined, the expression of PR genes has served as a reliable indirect indication for the activation of SAR (Ryals et al., 1996). Especially, expression of *PR-1a* (acidic PR-1) gene is most highly induced by TMV infection and exogenous application of salicylic acid (SA) in tobacco plants (Oh et al., 1999; Ward et al., 1991). Furthermore, *nahG* transgenic plants were unable to accumulate SA and thus resulting in defects in SAR and the expression of *PR-1* genes (Friedrich et al., 1995). Since *PR-1* is specifically induced by pathogen infection or SA treatment, *PR-1* gene has been commonly used as a molecular marker for SAR (Ryals et al., 1996).

In the previous study, Van de Rhee et al. (1990) reported that 0.9 kb of the *PR1a* promoter drives high levels of inducible *GUS* reporter gene expression by TMV infection or SA treatments. To further identify the important *cis*-acting DNA elements of *PR-1a* gene promoter in response to various stimuli, *PR-1a* promoter fused with β-glucuronidase (GUS) reporter gene construct has been subject to the promoter deletion analysis or mutagenesis approaches (Grüner et al., 2003; Strompen et al., 1998). For examples, residing within the -960 bp promoter region of the tobacco *PR-1a* gene, *as-1*-like motif has been shown to control GUS reporter gene expression in transgenic plants in response to TMV infection or SA treatment (Strompen et al., 1998). Several important *cis*-acting elements within the promoter of *PR-1a* gene responsive to pathogen or SA have been identified and the corresponding *trans*-acting factors have also been isolated (Rushton et al., 1996; Strompen et al., 1998). Pathogen and/or SA-induced proteins such as WRKY or TGA factors recognize *W*-box DNA sequence (TTGAC) or *as-1*-like motif (TGACG), which is present within the promoter of *PR-1a* gene. In addition, PcWRKY1 protein was shown to recognize the elicitor response elements (TTGAC) of the parsley *PR-1* gene (Rushton et

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al., 1996). TGA factor was demonstrated to bind to TGACG core DNA sequences within the -1.5 kb promoter region of the tobacco acidic *PR-1* gene (Strompen et al., 1998). Taken together, these data suggest that a *PR-1a* promoter is responsive to SA or to various signal molecules produced during pathogen attacks.

In addition to SA, other synthetic chemicals such as benzo(1,2,3)thiazole-7-carbothionic acid S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA) have been reported to induce the defense responses and SAR (Görlach et al., 1996; Métraux et al., 1991). When the BTH was sprayed on wheat plants at an early developmental stage, resistance was induced against several plant fungal diseases (Schurter et al., 1987). Therefore, the BTH was developed as the first plant activator agrochemical Bion® by Nobartis Crop Protection. The development of the new type of plant protection strategy has a significant impact on agriculture. Novel chemicals and natural products from various sources could be tested for the activity of plant resistance induction.

In this study, to develop a simple screening technique for the selection of plant defense-inducing agents from various microorganisms, we have constructed the -1.5 kb tobacco *PR-1a* promoter-*GUS* chimeric vector, and examined GUS reporter gene expression in transgenic tobacco plants in response to SA or BTH. In conclusion, we present a highly efficient chimeric *PR-1a* promoter-*GUS* reporter system for the screening of signal molecules involved in the regulation of the expression of *PR-1* gene and potentially induction of SAR.

Materials and Methods

Plant material and chemical treatments. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were grown in a controlled environment room at 25 ± 1°C with a photoperiod of 16 hr light. Four to six-week-old wild type or transgenic tobacco plants were used for chemical treatments. For the chemical treatments, transgenic plants were sprayed or emerged with 2 mM salicylic acid (SA), 0.3 mM benzol (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH). All the harvested leaves were frozen immediately in liquid nitrogen and stored at -70°C until RNA extraction.

DNA and RNA gel blot analyses. Genomic DNA was isolated from transgenic tobacco leaves as previously described (Oh et al., 2005). Twenty ìg of genomic DNA samples were digested with *Bam*HI. The digested genomic DNA was separated by electrophoresis in a 0.7% agarose gel, denatured, and blotted onto a nylon membrane (Amersham, USA). DNA blot hybridization was performed as previously described (Church and Gilbert, 1984), using a *gus* cDNA probe labeled with ^{32}[P]-dCTP. For RNA gel blot analysis, 20-μg of total RNA samples isolated from chemical-treated leaf tissues were size-fractionated in a formaldehyde-containing agarose gel and transferred onto a Nytran membrane (Amersham Pharmacia, USA) as described by Oh et al. (2005). *NtPR-1a* and *gus* cDNA probes were labeled with ^{32}[P]-dCTP using the Prime-a-Gene System (Promega, USA) according to the manufacturer's instructions, and then used for Northern blotting. Prehybridization and hybridization were performed at 42°C using 6 x SSPE containing 50% formamide, and the washing steps were performed as described by Oh et al. (2005). After the washing steps, the filters were exposed to X-ray film (Kodak, USA) with intensifying screens at -70°C for autoradiograph.

***PR-1a* promoter-*GUS* reporter genes construction and *Agrobacterium*-mediated transformation.** For the construction of the *PR-1a* promoter-*GUS* reporter gene, 20 ng of chromosomal DNA (*N. tabacum* cv. WI 38) was used for the amplification with primers [*Hin*dIII site (-1532 ~ -1510 region) 5′-GGAAGCTTGGACTAAGATATACGAGGATGTC-3′ and *BamH*I site (+7 ~ +31 region) 5′-GGGGATCCGACTATAGGATAAATGTTGTATCT-3′, Grüner and Pfitzner, 1994] under 60°C of annealing temperature. Amplification was performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min with 35 cycles. The amplified PCR product was run on a 1% agarose gel, and purified using gel extraction kit (Qiagen, Germany). The purified PCR fragment was cloned in pGEM-T Easy vector (Promega, USA) and the entire nucleotide sequences were determined. The 1.5 kb fragment in the pGEM-T Easy plasmid was also digested with the *Hin*dIII and *Bam*HI restriction enzymes and cloned into the binary vector pBI101 (renamed as *PR1a-GUS*). Leaf discs of *N. tabacum* cv. Samsun NN were transformed with *Agrobacterium tumefaciens* strain LBA4404 containing pBI121 or pBI101-derived vectors as previously described (Suh et al., 1998).

Each *Agrobacterium* cell was grown overnight in YEP (yeast extract 10 g/L, Bacto-Peptone 10 g/L, NaCl 5 g/L) medium containing 50 mg/L rifampicin, 50 mg/L kanamycin, and 100 μM acetosyringone. Fresh tobacco leaf discs were immersed in the bacterial suspension for 10 min, blotted on sterile paper towels, placed on basal MS agar medium (Murashige and Skoog, 1962), and incubated at 28°C in the dark for 2 days. Leaf discs were transferred to selection media containing 50 mg/L kanamycin. Ten *PR1a-GUS* independent tobacco transgenic lines (T_0) were regenerated and as were 5 independent lines containing pBI101 or pBI121 vector were obtained respectively. Primary transgenic tobacco lines (T_1) having a single copy of *PR1a-GUS* constructs was chosen by genomic DNA gel

blot analyses. The selected transgenic lines allowed to self-pollinating and T_2 seeds were harvested and tested for 100% germination on MS medium with kanamycin to select homozygote line. The selected T_2 line was used to investigate the defense induction activity in the following experiments.

GUS histochemical assay. The histochemical β-D-glucuronidase (GUS) assay was carried out as described by Jefferson et al. (1987). Transgenic tobacco plants were treated with 10% acetone, SA (2 mM) or BTH (300 μM) for 0, 24, 48, and 72 hr before GUS histochemical assay. Histochemical reactions with the substrate, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-Gluc) (Duchefa Biochemie, Netherlands), were incubated overnight at 37°C. After staining, tobacco seedling and leaves were rinsed in 70% ethanol for clearing, and then mounted for photograph.

Results and Discussions

Construction and development of a *PR-1a* promoter-*GUS* reporter system. Previously, the tobacco *PR-1a* promoter analysis has demonstrated that -1.5 kb or -0.9 kb upstream region of the *PR-1a* gene is sufficient to regulate *GUS* reporter gene expression in transgenic tobacco plants (Grüner and Pfitzner, 1994; Grüner et al., 2003; Strompen et al., 1998). In addition, Grüner and Pfitzner (1994) demonstrated that *GUS* reporter gene driven by the -1.5 kb *PR-1a* promoter region was stronger than by the -0.9 kb *PR-1a* promoter region in transgenic plants.

In order to develop the *PR-1a* promoter-*GUS* reporter system in tobacco, we amplified 1.5 kb fragment of *PR-1a* promoter, and constructed *PR-1a* promoter-*GUS* reporter gene. *Agrobacterium*-mediated transformation was employed to introduce the promoter-*GUS* fusion constructs into *N. tabacum* cv. Samsun NN plant as described in material and methods (Fig. 1). Primary transformants were allowed to self-fertilize, and progeny plants were identified through antibiotic resistance selection (100 mg/L kanamycin, data not shown). Ten independent transgenic lines were analyzed by DNA gel blot analysis with the *gus* probe, and 3 lines (*PR1a-GUS* # 5, 6, and 7 lines) showing a single insertion of the *GUS* gene were selected (Fig. 2A). Consequently, these selected plants were transferred to soil (T_1 generation) and analyzed for reporter gene expression in response to chemicals. The *PR1a-GUS*-expressing transgenic tobacco plants did not exhibit any apparent phenotypic abnormalities compared that of to the wild-type plants (data not shown).

To evaluate the effects of plant-defense inducing agents on *PR-1a* expression, we examined *PR-1a* transcript levels in tobacco leaves after treatment with SA or its derivative BTH. *PR-1a* transcript levels peaked between 24 to 48 hr after SA and BTH treatments, and its transcripts continued

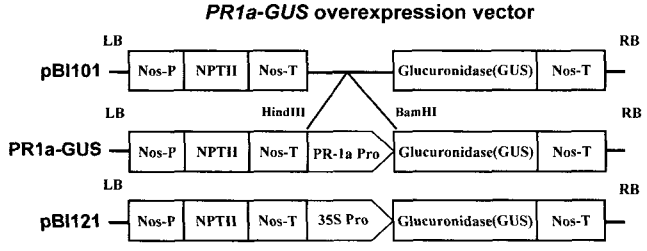

Fig. 1. Diagram of *pBI101, PR1a-GUS* and *pBI121* constructs. Tobacco plants (*N. tobacum* cv. Samsun NN) were transformed with the constructs pBI101, pBI121 (CaMV35S-GUS) and PR1a-GUS, respectively. Nos-P, nos promoter; NPTII, neomycin phosphotransferase; Nos-T, nos-terminator; 35S pro, CaMV 35S promoter; PR1a pro, 1.5 kb fragment of the pathogenesis-related protein-1a promoter; LB and RB, the left and right border of the T-DNA.

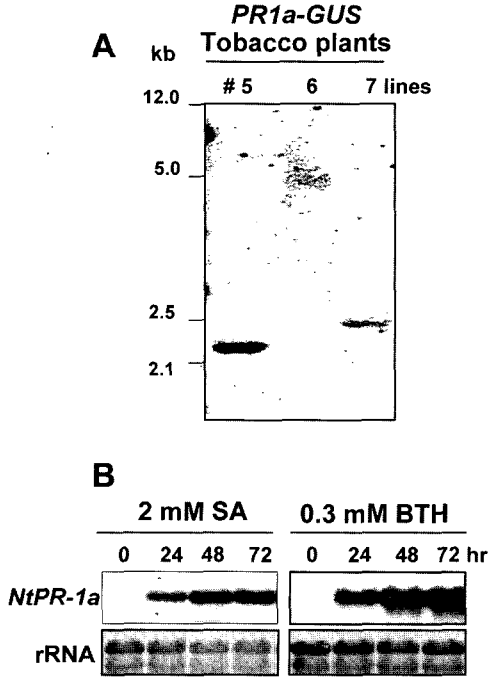

Fig. 2. Southern blot analysis of *PR1a-GUS* transgenic plants and RNA gel blot analysis of *PR-1* gene after treatment of SA or BTH. (**A**) Genomic DNA blot analysis of *PR1a-GUS* transgenic tobacco plants. Each lane was loaded with 20 μg of tobacco genomic DNA digested with *Bam*HI. The membranes were hybridized with ^{32}[P]-dCTP-labeled *gus* cDNA. The sizes of the molecular weight markers are indicated in kilobases to the left. (**B**) Tobacco plants were treated with SA or BTH for various time points. The treated leaves were harvested at the indicated time points and RNA gel blot analysis was performed as described in Materials and Methods. The ethidium bromide-stained gel is shown as an equal loading control.

to be expressed up to at least 72 hr (Fig. 2B). From these results, we confirmed that *PR-1a* is specifically induced by treatment of defense regulators such as SA or its analogous agent BTH (Ward et al., 1991).

GUS histochemical assay of *PR-1a* promoter-GUS transgenic tobacco plants by SA or BTH. To test the effects of plant defense-related signal molecules on *PR1a-GUS* reporter gene expression, we examined GUS activities and *GUS* transcripts levels in the three independent primary transformants after treatment with SA (T_1 generation seedlings, Fig. 3A). As shown in Fig. 3A, GUS reporter gene expression was clearly detected in *PR-1a* promoter-*GUS* seedlings after treatment of SA, but not in *pBI101* (minimal *TATA* elements-*GUS*). Furthermore, the level of *GUS* mRNA under SA treatment was correlated with the level of GUS expression activity in *pBI121* and *PR1a-GUS* transgenic plants (Fig. 3A). The # 5 line out of 3 *PR1a-GUS* transgenic plants were showed stable *GUS* expression. The verified homozygote T_2 line was used for further experiments. As positive control, seedlings containing *35S* promoter-*GUS* (pBI121) showed constitutive GUS expression with or without SA treatments (Fig. 3A).

We tested for the expression of the reporter *GUS* gene by treatments of SA or its analogous BTH in PR1a-GUS plants compared to non-transgenic, pBI101, and pBI121 transgenic plants (Fig. 3B and 3C). GUS activity started to be detected weakly in 24 hr after SA treatment and remained at significant levels until 72 hr post-treatment (Fig. 3B). Furthermore, we also examined GUS activity in the *PR1a-GUS* line (# 5) in 72 hr after BTH treatment (Fig. 3C). The *pBI121* transgenic plants showed constitutive GUS expression by any conditions (Fig. 3C). We observed that the level of GUS activity detected in BTH-treated or the lower, upper leaves in *PR1a-GUS* line # 5 T_2 plants (Fig. 3C). GUS activity was most strong in the BTH-treated leaves while the lower or upper leaves from the treated leaves showed weaker GUS staining. This demonstrates the fact that *PR-1* gene expression is mediated locally or systemically by SAR in plant (Glazebrook et al., 2003; Ryals et al., 1996). Previous studies from several groups had reported that about 0.9 kb or 1.5 kb of the *PR-1a* promoter region are sufficient to induce high-level expression of *PR-1a* promoter-*GUS* reporter gene constructs in transgenic tobacco plants in response to TMV infection, to treatment of the plants with SA and its analogues (Grüner and Pfitzner, 1994; Grüner et al., 2003; Strompen et al., 1998; Van de Rhee et al., 1990). We also demonstrate that GUS activity was highly responsive to exogenous application of SA and its analogue BTH in *PR1a-GUS* transgenic plants (Fig. 3).

In a course of development of high throughput screening

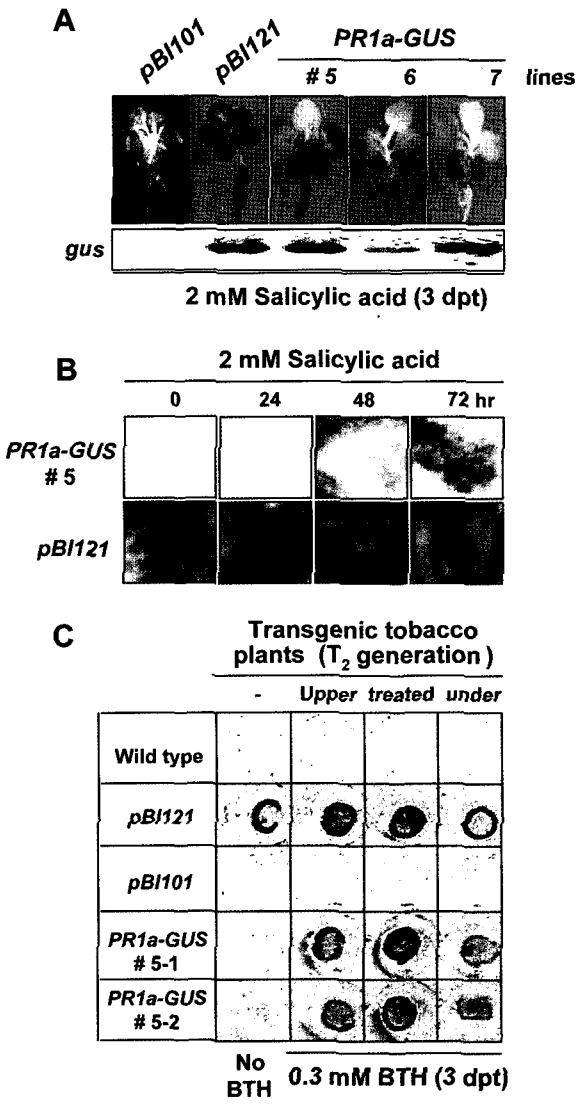

Fig. 3. GUS reporter gene expression in *PR1a-GUS* transgenic tobacco plants *PR-1a* after treatment with SA or BTH. (**A**) *Histochemical GUS staining and GUS gene expression in* PR1a-*GUS* transgenic tobacco seedlings after treatment with 2 mM SA solution. RNA gel blot analysis was carried out for *GUS* gene expression in *pBI101, pBI121*, and three *PR1a-GUS* transgenic plants after treatment with 2 mM SA. (**B**) GUS histochemical staining in *PR1a-GUS* (# 5) line and *pBI121* transgenic plants. The leaves were treated without or with 2 mM SA spray and GUS staining was made at 24, 48, and 72 hr after SA treatments. (**C**) GUS histochemical staining in non-transgenic, *pBI121, pBI101, and PR1a-GUS (# 5) transgenic plants treated with 0.3 mM BTH* for 3 day post-treatment (dpt). The GUS staining assay was performed as described by Jefferson et al. (1987).

(HTS) system to search for the novel plant activator from chemical library, cell suspension culture-based GUS expression assay was investigated by incubating various concentrations of SA and BTH with the transgenic tobacco cells in 96-well format. Although the partial weak GUS

expression was investigated 72 hr after incubation and 12 hr after GUS staining process, the result was not consistent. Therefore, optimization of cell suspension culture condition to maintain the stable physiological status is probably necessary to develop the practical HTS system.

In summary, we present a simple screening system using *PR-1a* promoter-*GUS* transgenic tobacco plants. Using GUS histochemical assay as a reporter system, GUS activity was locally or systemically detected by *PR-1a* promoter in tobacco leaves with the SA or BTH treatments. Hence, our screening system based on transgenic plants provides the valuable selection method of the defense-inducing synthetic compounds and potentially metabolites produced by micro-organisms, and for advances in the large-scale screening of many chemical agents.

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