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Technical Note

High-throughput screening for plant defense activators using a *β-glucuronidase*-reporter gene assay in *Arabidopsis thaliana*

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Abstract To develop a screening system for plant defense activators, which are novel substances that protect plants by enhancing their inherent disease-resistance mechanisms, we utilized a GUS histochemical staining assay using promoters of the defense-related genes, *PR-1* and *PDF1.2*. We can perform about 1,000 screenings per week per person by this high-throughput screening method. This GUS assay for plant defense activator candidates was evaluated by QRT-PCR analysis to elucidate the functions of the plant defense activators in detail. In the present preliminary screening, we evaluated two hundred chemicals chosen at random. Some chemicals induced GUS activity in a *PR-1* promoter::*GUS* transformant, i.e., abietic acid, allose, glycine, and thymol. The induction of *PR-1* expression by the treatments with these chemicals was confirmed using QRT-PCR. The foliar treatment with abietic acid 1 d prior to inoculation with the fungal pathogen *Colletotrichum higginsianum* led to a significant reduction of necrotic surface area compared with distilled water treated controls, as observed 6 d after inoculation. These results suggest that this GUS histochemical staining assay is an effective and available screening system for plant defense activators.

Key words: Abietic acid, *β-glucuronidase*, high-throughput screening system, histochemical staining, plant defense activator.

Systemic acquired resistance (SAR) is an inducible defense mechanism that plays an important role in defending plants from attack by pathogens (Chester 1933; Durner et al. 1997). SAR in plants is a whole-plant defense response that occurs following an earlier localized exposure to a pathogen. In induced resistance processes, more than one biochemical pathway appears to be activated, on the basis of the requirement of different signal transduction pathways depending on salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). SAR has been well characterized in tobacco and *Arabidopsis thaliana*, and SA was found to be the signaling molecule (Gaffney et al. 1993; Delaney et al. 1994). Several synthetic compounds that induce SAR and the expression of various SAR-associated marker genes, including *PR* genes, have been identified. For example, 2,6-dichloroisonicotinic acid (INA), benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), and *N*-cyanomethyl-2-chloroisonicotinamide (NCI) induce SAR by stimulating the signal transduction pathway for SAR development at the same point or downstream of SA accumulation (Friedrich et al. 1996; Lawton et al. 1996; Nakashita et al. 2002; Yasuda et al. 2003). In contrast, probenazole (PBZ) and its derivative, benzisothiazole (BIT), stimulate the SAR signaling pathway upstream of SA accumulation (Yoshioka et al. 2001). The non-protein amino acid β-aminobutyric acid (BABA) has been shown to protect *A. thaliana* against some pathogens through the activation of defense mechanisms such as callose deposition and hypersensitive cell death (Jakab et al. 2001; Zimmerli et al. 2000). Although these synthetic compounds activate plant defense mechanisms, they do not have any direct activity against pathogens, and hence are classified as chemical or plant defense activators (Friedrich et al. 1996; Kessman et al. 1994). A plant defense activator is a chemical inducer of SAR mainly developed to protect plants against pathogen attack. The plant defense activators offer several advantages over conventional fungitoxic compounds. (1) Using SAR for plant protection is regarded as environmentally friendly, since only one application at the beginning of the growing season is sufficient to protect the plants for the entire year. (2) Nonfungitoxic compounds which regulate

Abbreviations: *GUS*, *β-glucuronidase*; ET, ethylene; JA, jasmonic acid; SA, salicylic acid; SAR, systemic acquired resistance.

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host resistance are likely, in many cases, to be active at extremely low levels. (3) Plant defense activators that enhance host defense activity are less likely to encounter fungal resistance than are many conventional systemic fungicides. Although plant defense activators are potentially very useful for crop disease control and management, their identification is challenging because they lack any direct toxic effects against pathogenic fungi and bacteria. For plant–pathogen interactions, large-scale screening is currently limited by the time and space required for plant growth. Schreiber et al. (2008) devised a high-throughput assay using a model pathosystem between *A. thaliana* and *Pseudomonas syringae*. This assay screened for compounds that reduce bacterial virulence in the plants. In this study, we developed a high-throughput screening system for plant defense activators by monitoring the induction of the plant immune system using a *β-glucuronidase* (*GUS*) reporter gene assay in standard 48-well plates.

Previously, we had developed an evaluation system using *A. thaliana* transformants to readily monitor the expression profile of defense-related genes in response to chemical treatment (Narusaka et al. 2006). In this study, we used the *A. thaliana PR-1* gene as a marker for the SA-dependent signal transduction pathway and *PDF1.2* as a marker for the ET/JA-dependent signal transduction pathway. We fused the promoter regions 1.29 and 1.25 kbp upstream of the start codons of *PR-1* and *PDF1.2*, respectively, to the *GUS* gene. T_3 homozygous transgenic *A. thaliana* plants harboring the *PR-1* and *PDF1.2* promoter-*GUS* fusions were generated to investigate regulated gene expression. To evaluate the screening system, the appropriate volume of reagent solutions, i.e., 10 or 100 ppm of sodium salicylate (SANa), ethephon or distilled water (DW) was added to a 48-well microtiter plate. The transgenic *A. thaliana* plants were grown in soil for 28 d in a growth chamber at 22°C under a 12 h light/12 h dark cycle. Then one detached leaf was placed into each well containing the reagent solution, and the microtiter plate was kept at 22°C under dim light. Twenty-four or forty-eight h later, the *GUS* reporter gene assay was performed using a standard protocol for histochemical staining (Jefferson et al. 1986; Nakashima et al. 1997). To evaluate the GUS histochemical staining assay, we performed the assay to detect the specific activation of *PR-1* and *PDF1.2* promoters after the treatment with SANa and ethephon.

The GUS staining scores (GSS) were assigned based on a relative amount of GUS expression as determined histochemically for blue staining (Figure 1). The GSS was rated on a scale from 1 to 5, with a score of 0 indicating that no blue flecking was observed. A GSS of 1 was assigned when small blue flecks were visible; 2 indicated that the leaf was slightly stained to light blue; 3 that a leaf was partially stained to blue; 4 that a whole leaf was stained to blue; and 5 that a whole leaf was stained to dark blue along with the reaction solution dyed in blue.

Table 1. GUS staining scores in *PR-1p*::*GUS* and *PDF1.2p*::*GUS* transformants treated with chemicals

Treatment	GUS staining score			
	PR-1p::*GUS*		*PDF1.2p*::*GUS*	
	100 ppm	10 ppm	100 ppm	10 ppm
distilled water (24 h)[a]	0	0	0	0
distilled water (48 h)	0	0	0	0
sodium salicylate (24 h)	4	2	0	0
sodium salicylate (48 h)	4	3	0	0
ethephon (24 h)	0	0	died[b]	5
ethephon (48 h)	0	0	died[b]	4
abietic acid (24 h)	1[b]	1	0	0
allose (24 h)	1	0	0	0
glycine (24 h)	2	1	0	0
thymol (24 h)	2	0	0	0

[a] Incubation time for chemical treatment.
[b] The treatment with these reagents caused chemical poisoning to the detached leaf.

The transformants with the *PR-1* promoter::*GUS* gene fusion (*PR-1p*::*GUS*) showed GSS 2 and 4, respectively, after the treatment with 10 and 100 ppm SA for 24 h. However, GSS of 0 was obtained for *PDF1.2* transformants (*PDF1.2p*::*GUS*) under these conditions (Table 1). The *PR-1p*::*GUS* transformant showed GSS 3 and 4, respectively, after the treatment with 10 and 100 ppm SANa for 48 h. Under the same conditions, GSS was 0 for the *PDF1.2p*::*GUS* transformant. The *PDF1.2p*::*GUS* transformant displayed a GSS of 5 after the treatment with 10 ppm ethephon for 24 h and a GSS of 4 for 48 h. The treatment with 100 ppm ethephon caused chemical poisoning to the detached leaf. These results suggest that GUS activity is induced by the treatment with active chemicals under this condition. In a previous study, we used a GUS fluorescence assay to detect the specific activation of *PR-1* and *PR-4* promoters (Narusaka et al. 2006). In this study, we used a GUS histochemical staining assay to detect the specific activation of *PR-1* and *PDF1.2* promoters. The advantage of this method over the previous one is that a large amount of work can be done easier than for GUS fluorescence assay.

To evaluate this screening system using the GUS histochemical staining assay, we evaluated two hundred other chemicals chosen at random (general analytical reagents; standard laboratory reagents) by the same GUS assay screening system and evaluated the results with QRT-PCR. The results showed that some chemicals induced GUS activity in the *PR-1p*::*GUS* transformant, i.e., abietic acid, allose, glycine, and thymol (Table 1). The results showed that the transformants with the *PR-1p*::*GUS* showed GSS 1 after the treatment with 10 and 100 ppm abietic acid for 24 h. The treatment with 100

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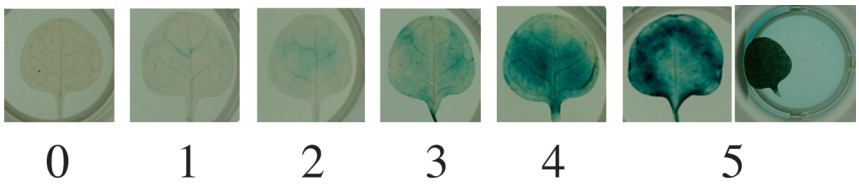

Figure 1. Evaluation system using *A. thaliana* transformants to monitor the expression profile of defense-related genes in response to chemical treatments. The GUS staining scores (GSS) were assigned on the basis of rating of staining to blue; 0, no blue flecking; 1, small blue flecks; 2, slightly staining to light blue; 3, partially staining to blue; 4, whole leaf staining to blue; 5, whole leaf staining to dark blue along with the reaction solution dyed in blue.

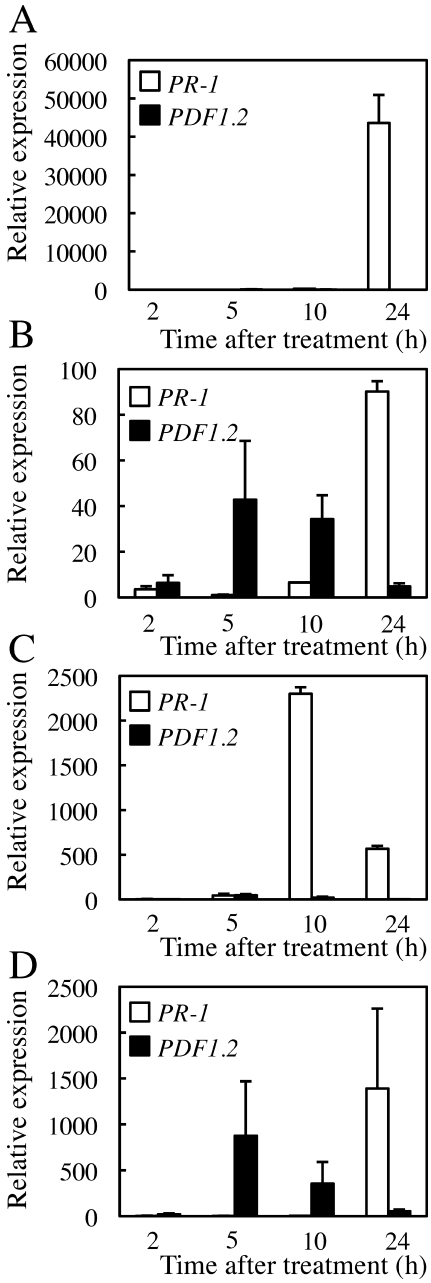

Figure 2. *PR-1* and *PDF1.2* expression over time after treatments with chemicals in Col-0 wild-type plants of *A. thaliana*. Plants were grown in soil for 28 d in a growth chamber at 22°C under a 12 h light/12 h dark cycle, then sprayed with 100 ppm abietic acid (A), allose (B), glycine (C), and thymol (D). The expression of *PR-1* and *PDF1.2* was monitored by QRT-PCR. The experiment was repeated at least three times. Bars indicate SE.

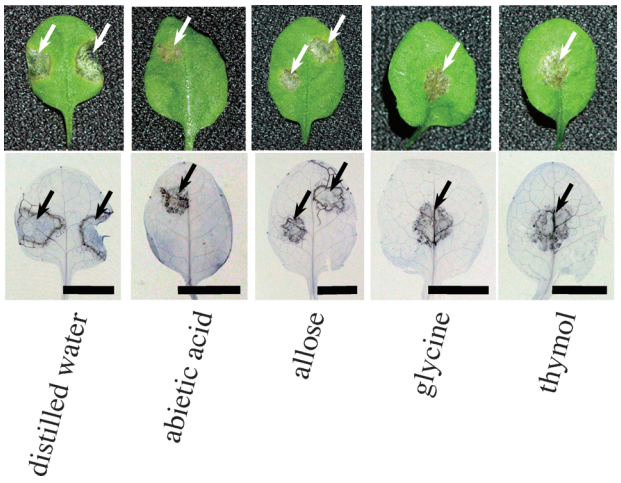

Figure 3. Protection of *A. thaliana* by chemicals against infection with *C. higginsianum*. Leaves of 28 day-old plants (12 h light/12 h dark cycle) were sprayed with distilled water or 100 ppm abietic acid, allose, glycine, and thymol prior to drop inoculation with one to two 5-μl drops of a spore suspension of *C. higginsianum* (5×10^5 spores ml^{-1}) on each leaf. The leaves were harvested at 6 dpi and stained with trypan blue. Arrows indicate the inoculation sites and lesions. Bars=0.5 cm. Each picture shows a representative of three independent experiments.

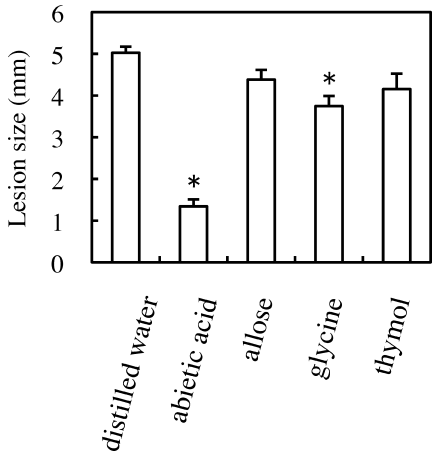

Figure 4. Lesion diameters on the leaves treated with chemicals, 6 days after inoculation with *C. higginsianum*. Leaves of 28 day-old plants (12 h light/12 h dark cycle) were sprayed with distilled water or 100 ppm abietic acid, allose, glycine, and thymol prior to drop inoculation with one to two 5-μl drops of a spore suspension of *C. higginsianum* (5×10^5 spores ml^{-1}) on each leaf. The data are obtained from more than 10 plants for each treatment. The experiment was repeated at least three times. Bars indicate SE. Asterisks indicate a statistical significance from distilled water controls (Dunnett's method, $P<0.05$).

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ppm abietic acid for 24 h decreased a relative amount of GUS expression because the treatment with the reagent caused chemical poisoning to the detached leaf. The *PR-1p*::*GUS* transformant showed GSS 1 and 2, respectively, after the treatment with 10 and 100 ppm glycine for 24 h. Under the same conditions, GSS was 0 for the *PDF1.2p*::*GUS* transformant. The *PR-1p*::*GUS* transformant showed GSS 1 and 2, respectively, after the treatment with 100 ppm allose and thymol for 24 h.

The stability of GUS protein is well known and can often give misleading results when examining expression over time because of the long turn-over time (Taylor 1997). For this reason the level of *PR-1* and *PDF1.2* expression were also measured by QRT-PCR to confirm histochemical staining (Moon and Callahan 2004). Whole *A. thaliana* plants (Col-0 wild-type) were sprayed with 100 ppm abietic acid, allose, glycine, or thymol. The plants were then returned to the growth chamber. The plants were harvested 2, 5, 10, and 24 h after these treatments and then frozen immediately in liquid nitrogen until further analysis. Total RNA was isolated and the induction of *PR-1* and *PDF1.2* were evaluated by QRT-PCR (Figure 2). Total RNA was isolated by RNA tissue Kit II, according to the manufacturer's instructions (Fujifilm, Tokyo, Japan). First-strand cDNA was synthesized from 500 ng of total RNA treated with DNase, using PrimeScript RT reagent Kit, according to the manufacturer's instructions (Takara, Otsu, Japan). QRT-PCR was performed using SYBR Green PCR Master Mix (Takara, Otsu, Japan) with the first-strand cDNA as a template with an MJ Opticon (BIO-Rad Laboratories, Hercules, CA, USA). QRT-PCR mixtures consisted of 1x SYBR Green I PCR Master Mix and 200 nM (each) sense and antisense primers. Following a preliminary denaturation step at 95°C for 10 s, the reaction mixtures were subjected to 40 cycles at 95°C for 5 s and at 65°C for 20 s. The target sample copy number was averaged for two reactions, and the biological experiment was repeated three times. The expression of the *CBP20* gene was used for normalization as a standard control gene. Nucleotide sequences of gene-specific primers for each gene are as follows: *CBP20* (At5g44200; forward primer 5′-TGTTTCGTCCTGTT-CTACTC-3′, reverse primer 5′-ACACGAATAGGCCG-GTCATC-3′); *PR-1* (At2g14610; forward primer 5′-CCCACAAGATTATCTAAGGGTTCAC-3′, reverse primer 5′-CCCTCTCGTCCCACTGCAT-3′) (Jirage et al. 2001); *PDF1.2* (At5g44420; forward primer 5′-CCATCATCACCCTTATCTTCGC-3′, reverse primer 5′-TGTCCCACTTGGCTTCTCG-3′). The *PR-1* expression strongly increased 24 h after the abietic acid treatment (Figure 2A), but the transcription level of *PDF1.2* was low during the treatment. The *PR-1* expression peaked at 10 h after the glycine treatment (Figure 2C) but the *PDF1.2* transcription was low during the treatment. The *PR-1* expression peaked at 24 h after the thymol treatment (Figure 2D) but the expression of the *PDF1.2* gene increased with a peak at 5 h after the treatment. The transcription levels of both *PR-1* and *PDF1.2* were low during the allose treatment (Figure 2B).

To understand the nature of the resistance induced by these chemicals, cytological observations were performed on infection sites of a virulent fungal pathogen *Colletotrichum higginsianum* strain MAFF305635 (MAFF Genebank, Japan) in *A. thaliana* leaves (Col-0) (Figure 3, 4). Plants were sprayed with DW or 100 ppm abietic acid, allose, glycine, and thymol 1 d prior to drop inoculation with one to two 5-μl droplets containing 5×10^5 spores ml^{-1} of *C. higginsianum*. The foliar treatment with abietic acid 1 d prior to the inoculation led to a significant reduction of necrotic surface area compared with the DW treated controls, as observed 6 d after inoculation. On the contrary, the foliar treatments with glycine and thymol led to a slight reduction of the necroses, but the allose treatment had no effect on the reduction of necroses. The antimicrobial activity of abietic acid was reported previously (Himejima et al. 1992). The antifungal activity of the compound may lead to inhibition of growth and reproduction of the pathogen in its parasitic phases. The compound may act directly on the pathogen to prevent it from becoming established in the plant tissue or from causing disease. The disease control may involve both direct fungitoxicity and enhanced host resistance mechanisms. For examples, these combination effects were shown by metalaxyl (Barak and Edgington 1983) and fosetyl-Al (Andreu et al. 2006).

The results of this study show that candidate plant defense activators can be screened easily by monitoring *PR-1* and *PDF1.2* promoter activity after various chemical treatments. In addition, the plant defense activators can be classified into those activating the SA-signaling pathway and those activating the JA/ET-signaling pathway. Furthermore, antifungal activity of these chemicals should be evaluated. We will also evaluate the effects of candidates for some host-parasite interactions in the future.

In assays using methods such as QRT-PCR, Northern hybridization, and microarray, samples should be taken at several points because the mode of action for almost all chemicals is unknown. On the other hand, the GUS assay does not allow quantification because the GUS protein is stably accumulated in transgenic plants after chemical treatment. Therefore, sampling at one point is enough with this assay to analyze gene induction by unknown chemicals. In this study, we used the GUS histochemical staining assay 24 and 48 h after treatment. In high-throughput screening, it is important to evaluate only a few processes. The high-throughput screening method allows one person to perform more than 1,000

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screenings per week. The GUS assay for plant defense activator candidates used in this study needs to be studied further by QRT-PCR or microarray analyses to elucidate the functions of the plant defense activators in detail. The protective effect also needs to be evaluated by inoculation with plant pathogens.

This screening can monitor the induction of SA- and ET/JA-induced resistance. The knowledge gained here will enable the development of new plant defense activators and will offer novel perspectives for engineering durable resistance in crop plants. This approach is effective for large-scale screening of agrochemical plant defense activator candidates.

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