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Microarray Note

A model system to screen for candidate plant activators using an immune-induction system in *Arabidopsis*

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Abstract To develop a screening system for plant activators, which are novel substances that protect plants by enhancing their inherent disease-resistance mechanisms, we utilized a *GUS* reporter gene system using promoters of the defense-related genes, *PR-1* and *PR-4*. To validate the strategy, we performed subsequent analysis using an *Arabidopsis* microarray consisting of 1200 full-length cDNA clones representing putative defense-related and regulatory genes. Benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester and 2,6-dichloroisonicotinic acid activated plant defense responses via the salicylic acid (SA)-dependent signaling pathway, and β-aminobutyric acid triggered a primed state in the plant that enables more efficient activation of the SA-, jasmonic acid- and ethylene-signaling pathway. These results suggest that this novel system can be used to screen for candidate plant activators.

Key words: Microarray, plant activator, promoter, screening system.

Plants are continually challenged by pathogens, such as fungi, bacteria, and viruses in nature. However, only a few pathogens actually infect the plant and cause damage. Many plants defend themselves against fungi and other microbial pathogens by inducing both localized and systemic resistance responses. These responses are governed by hormonal regulation, in which salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) can each contribute.

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 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). The signal transduction pathway downstream of SA leads to the expression of a number of pathogenesis-related (*PR*) genes, such as *PR-1* and *β-1,3-glucanase* (Ryals et al. 1996). JA also modulates resistance to pathogens and insects as well as other physiological events such as fruit ripening and senescence. Wounding and methyl jasmonate (MeJA) treatment are known to induce the expression of *LOX2*, *AOS*, *VSPs*, *Thi2.1*, *PDF1.2* and various other stress-related genes (Creelman and Mullet 1995; Benedetti et al. 1995; Epple et al. 1995; Penninckx et al. 1996). ET regulates many aspects of plant growth, development and senescence (Yang and Hoffman 1985) and has also been implicated in disease resistance and disease susceptibility (Feys and Parker 2000). ET has also been shown to work synergistically with JA to activate induced systemic resistance (ISR) and *PDF1.2* gene expression in *Arabidopsis* (Pieterse and van Loon 1999).

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

Abbreviations: BABA, β-aminobutyric acid; BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; cDNA, complementary DNA; ET, ethylene; GUS, β-glucuronidase; INA, 2,6-dichloroisonicotinic acid; JA, jasmonic acid; SA, salicylic acid; SAR, systemic acquired resistance.
This article can be found at http://www.jspcmb.jp/

shown to protect *Arabidopsis* against some pathogens through 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 are hence classified as chemical activators or plant activators (Friedrich et al. 1996; Kessman et al. 1994). Plant activators are potentially very useful for crop disease control and management. However, their identification is challenging because they lack any direct toxic effects against pathogenic fungi and bacteria. In this study, we developed a model screening system for plant activators by monitoring the induction of the plant immune system and changes in expression of pathogen-responsive genes.

We developed a system using *Arabidopsis* transformants to readily monitor the expression profile of defense-related genes in response to chemical treatment. We used the *Arabidopsis* gene *PR-1* as a marker for the SA-dependent signal transduction pathway, and *PR-4* as a marker for the JA/ET pathway. We used 1.29 kbp and 1.5 kbp regions upstream from start codon (ATG) of *PR-1* and *PR-4* gene as a promoter region respectively, because most *Arabidopsis* promoters use *cis*-acting elements located within the first 1.0 kb and most *Arabidopsis* 5′ untranslated sequences are less than 150 bp (Maleck et al. 2000). T_3 homozygous transgenic *Arabidopsis* plants harboring the *PR-1*- or *PR-4*-promoter fused to the *β-glucuronidase* (*GUS*) gene were generated to investigate the regulated gene expression. The transgenic *Arabidopsis* plants were grown in soil for 28 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle. Then they were applied a foliar spray of 2.5 mM SA, 0.1 mM MeJA, 1 mM ethephon (as ethylene treatment), 0.5 mM BTH, 10 mM BABA, 1 mM INA, 2.5 mM *p*-hydroxybenzoic acid (4HBA), distilled water (DW) or DW plus 1% acetone (DW+A). Twenty-four h later, the *GUS* reporter gene assay was performed using a standard protocol (Jefferson et al. 1986). After the treatment with SA and MeJA or ethephon, we performed a GUS assay to detect specific activation of *PR-1* and *PR-4* promoters, respectively. The expression patterns were similar to those of endogenous *PR-1* or *PR-4* mRNAs (data not shown).

The transformant with the *PR-1* promoter::*GUS* gene fusion showed a 130.3-fold increase in GUS activity after treatment with 2.5 mM SA (Figure 1A). The transformant with the *PR-4* promoter::*GUS* gene displayed a 7.4-fold and 4.5-fold increase in GUS activity after treatment with 1 mM ethephon and 0.1 mM MeJA, respectively (Figure 1B). Similar treatment with an inactive SA analogue, 4HBA, did not affect promoter activity. These results suggest that GUS activity is not induced by treatment with inactive chemicals under this condition. The transformant with the *PR-1*

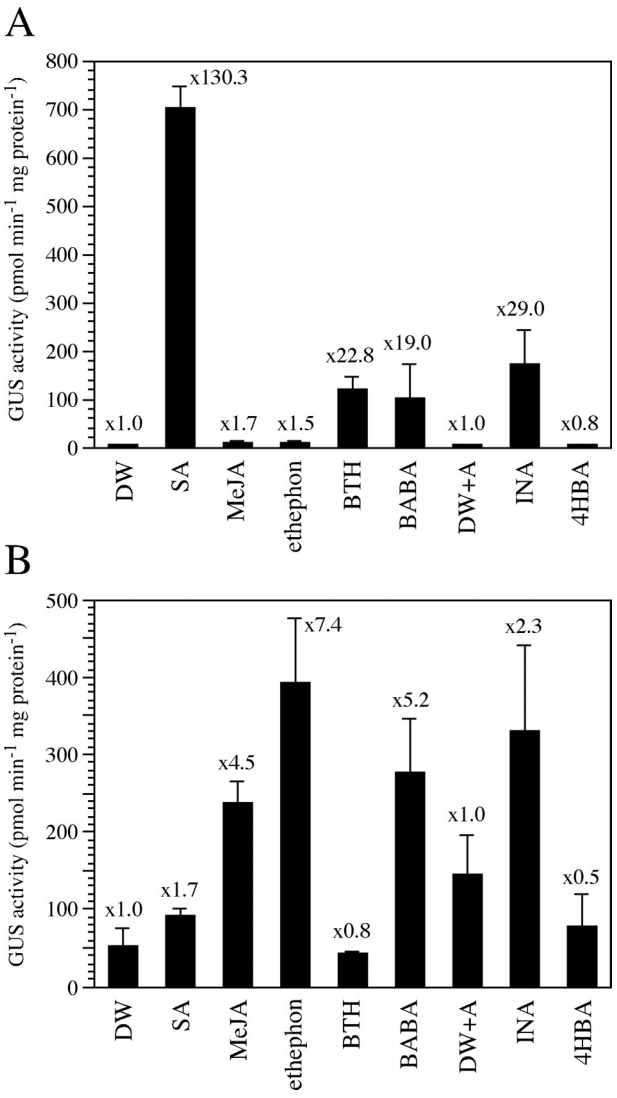

Figure 1. Plant activator-inducible expression of GUS activity in transgenic *Arabidopsis* plants carrying either the *PR-1* or *PR-4* promoter::*GUS* fusion gene, 24 h after treatment. The T_3 homozygous transgenic *Arabidopsis* plants with the *PR-1* (A) or *PR-4* (B) promoter::*GUS* fusion gene were grown in soil for 28 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle, and sprayed with 2.5 mM SA, 0.1 mM MeJA, 1 mM ethephon, 0.5 mM BTH, 10 mM BABA, 1 mM INA, 2.5 mM 4HBA, distilled water (DW) or DW plus 1% acetone (DW+A). Ratios indicate the multiplicities of expression compared with the value obtained with DW (SA, MeJA, ethephon, BTH, BABA) or DW plus 1% acetone (INA, 4HBA) treatments. The values of GUS activity are the averages of values obtained from more than 21 plants per treatment. Bars indicate SE.

promoter::*GUS* gene showed a 29.0- and 22.8-fold increase in GUS activity after 1 mM INA and 0.5 mM BTH treatment, respectively. In contrast, the levels of GUS activity in the transformant with the *PR-4* promoter::*GUS* gene were low after treatment with INA or BTH (Figure 1). After treatment with 10 mM BABA, the *PR-1* promoter::*GUS* transformants showed a 19.0-fold increase of GUS activity (Figure 1A) whereas the *PR-4* promoter::*GUS* transformants showed a 5.2-fold increase of GUS activity (Figure 1B), indicating that

BABA activates both the SA and JA/ET signaling pathways. The results of this study showed that candidate plant activators can be screened easily by monitoring *PR-1* and *PR-4* promoter activity after various chemical treatments, and that the plant activators could be classified into those activating the SA-signaling pathway and those activating the JA/ET-signaling pathway. This screening system needs to be developed using additional defense-related genes to elucidate further the functions of the plant activators.

The tobacco *PR-1a* gene, which is strictly up-regulated after activation of the SA-dependent signal transduction pathway, has been used as a marker to monitor chemical activation of this pathway, and transgenic tobacco BY-2 cells harboring the *PR-1a* promoter-*luciferase* fusion gene have been suggested to be useful for the study of defense gene expression (Ono et al. 2004). This system may also be used to screen for plant activators after modification for high through-put screening procedures (Ono et al. 2004; Ogura et al. 2005). Then, we will need an assay system to screen the candidate plant activators identified in the cell culture for activity *in planta*.

Previously, the characteristics of most plant activators were assessed by the expression of only a limited number of genes at a time. Here, we have adopted a more comprehensive approach that generates information about the expression of large numbers of genes simultaneously using an *Arabidopsis* microarray consisting of 1200 (1.2 K) full-length cDNA clones representing putative defense-related and regulatory genes.

A total of 1.2 K potential biotic and abiotic stress-related genes were selected from the genes covered by the *Arabidopsis* 7 K array (RIKEN, Japan) and *Arabidopsis* oligo microarray (Agilent Technologies, USA) for this study (Seki et al. 2002; Narusaka et al. 2003a; 2003b; 2004a; 2004b; 2005). To gain further information on the characterization of plant activators in the defense response, we studied their effect on the expression of the 1.2 K stress-related genes in our microarray.

Full-length cDNA microarray analysis was carried out essentially as reported previously (Seki et al. 2001; 2002; Narusaka et al. 2003a; 2003b; 2004a; 2004b; 2005). *Arabidopsis* wild-type plants (ecotype Columbia; Col-0) were grown in soil for 28 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle. *Arabidopsis* plants were applied a foliar spray with 5 mM SA, 0.1 mM MeJA, 1 mM ethephon, 0.5 mM BTH, 10 mM BABA and 1 mM INA. Leaves were harvested at 2, 5, 10 and 24 h after treatment, snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was isolated as described previously (Narusaka et al. 2004a). To ensure biological reproducibility of the microarray results, we replicated the experiment three to four times with between thirty and seventy plants per sample. To obtain sufficient material for the experiments, we pooled samples from replicate experiments prior to RNA extraction. In addition, we conducted two to three independent microarray analyses with the same RNA for reproducibility of handling. The results demonstrated a high degree of correlation in fold change values between the different data sets.

We regarded cDNAs with expression ratios (treated/untreated) five-fold greater than the expression ratio of the lambda control template DNA fragment (TX803; Takara, Kyoto, Japan) at one or more time points to be up-regulated, compound-inducible genes (Narusaka et al. 2004a; 2005; Seki et al. 2001; 2002). Untreated Col-0 plants grown under the same conditions as the treated plants were used for calculations of fold induction for all treatments including the water control. Image analysis and signal quantification were performed with a ScanArray Express version 3.0 (PerkinElmer, MA, USA). The median of 80 signal values obtained from the lambda control template DNA fragment was used as an external control to equalize hybridization signals generated from different samples. Gene-clustering analysis was performed with GeneSpring ver. 6.2 (Silicon Genetics, San Carlos, CA, USA).

The microarray data in this study is available with Gene Expression Omnibus (GEO) at http://www.ncbi.nih.gov/geo/ (See the figure legend in Fig. 2). To investigate the relationship between experiments, two different methodologies were performed in this study. First, the relation between experiment pairs was analyzed by means of Pearson's correlation coefficient using the software GeneSpring ver. 6.2 (Table 1). The absolute value of correlation coefficient is large when there is little variation within the groups compared to variation among group means. The results suggest that the correlation coefficient between INA- and SA-treatments was high between the two (INA 2 h and SA 5 h, INA 5 h and SA 10 h, INA 10 h and SA 10 h, INA 24 h and SA 24 h), and the weak correlation between INA- and ethephon-treatments was shown. In contrast, the correlation coefficient between INA- and MeJA-treatments is below 0.2, and therefore it suggests that their pairs are not close. The correlation coefficient between BTH- and SA-treatments indicates that two experiments are closer than other experiments (MeJA/ethephon), although the correlation coefficient between BTH- and ethephon-treatments is high 2 h and 24 h after BTH-treatment. The correlation coefficient between BABA- and SA/ethephon-treatments was high and the pairs are close. In contrast, the results indicate that the correlation coefficient between BABA- and MeJA-treatments is low and the pairs are not close.

Second, the ratios of expression levels of treated

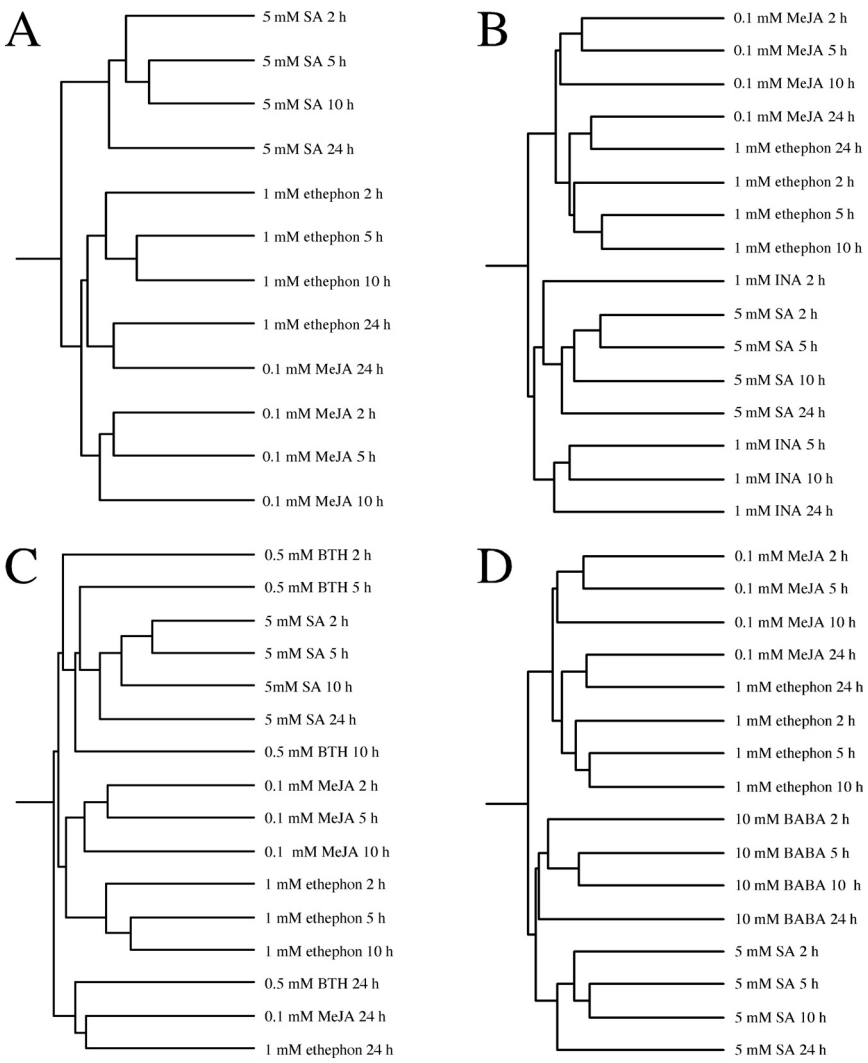

Figure 2. Hierarchical clustering of gene expression data in *Arabidopsis* Col-0 plants treated with plant activators. *Arabidopsis* plants were grown in soil for 28 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle, and treated with 5 mM SA, 0.1 mM MeJA, 1 mM ethephon, 0.5 mM BTH, 10 mM BABA, and 1 mM INA. Total RNA was extracted from *Arabidopsis* leaves at 2, 5, 10 and 24 h. Using a cDNA microarray, 241, 134 and 220 genes with expression ratios (treated/untreated) of more than five-fold at least at one time point were regarded as INA, BTH or BABA-inducible genes, respectively. (A) Hierarchical clustering of 1.2 K genes in response to treatment with SA, MeJA or ethephon. (B) Hierarchical clustering of 241 genes in response to the treatment with INA and treatment with SA, MeJA or ethephon. (C) Hierarchical clustering of 134 genes in response to the treatment with BTH and treatment with SA, MeJA or ethephon. (D) Hierarchical clustering of 220 genes in response to the treatment with BABA and treatment with SA, MeJA or ethephon. The lengths of the branches on the ordinate show the correlation between the treatments. Short branches indicate a closer correlation than long branches. The microarray data are available with Gene Expression Omnibus (GEO) at http://www.ncbi.nih.gov/geo/ (Accession No. GSM85771, GSM85772, GSM85773, GSM85774 for SA treatment; GSM85783, GSM85784, GSM85785, GSM85786 for MeJA treatment; GSM85236, GSM85624, GSM85625, GSM85663 for ET treatment; GSM85664, GSM85665, GSM85666, GSM85667, GSM85668, GSM85669 for INA treatment; GSM85779, GSM85780, GSM85781, GSM85782, GSM86106 for BTH treatment; GSM85775, GSM85776, GSM85777, GSM85778 for BABA treatment.

versus control Col-0 plants were $\log_2$ transformed and subjected to hierarchical clustering using GeneSpring ver. 6.2 (Figure 2A). On the basis of the altered expression patterns after SA, MeJA and ethephon treatments, we categorized these treatments into three groups. Expression analyses with the cDNA microarray indicated that changes in expression caused by ethephon were more similar to those caused by MeJA than SA.

Treatment of Col-0 plants with SA, MeJA and ethephon induced the expression of 282, 159 and 246 genes, respectively. There were 241 genes up-regulated by treatment with INA, including some genes known to be important for the SA-signaling pathway such as *PR-1* and *PR-5* and the increase of mRNA in Col-0 plants treated with INA peaked at 5 h. The 241 genes were subjected to clustering by treatments (INA, SA, MeJA and ethephon) and the data in Figure 2B shows the results of the cluster analysis of expression profiles. The dendrogram of Figure 2B indicates the relationship among experiments across all 241 genes included in the cluster analysis. The expression profiles of stress-inducible genes after treatment with INA resembled

Table 1. The correlation coefficient between experiment pairs on microarray analyses.

Treatment	SA 2 h	SA 5 h	SA 10 h	SA 24 h	Ethephon 2 h	Ethephon 5 h	Ethephon 10 h	Ethephon 24 h	MeJA 2 h	MeJA 5 h	MeJA 10 h	MeJA 24 h
INA 2 h	0.677	0.705	0.674	0.565	0.514	0.485	0.435	0.126	0.211	0.153	0.0596	−0.0472
INA 5 h	0.366	0.463	0.512	0.511	0.299	0.559	0.428	0.195	0.00376	0.0377	0.0303	−0.101
INA 10 h	0.507	0.612	0.74	0.696	0.373	0.575	0.569	0.294	0.0222	0.075	0.0724	−0.0673
INA 24 h	0.354	0.419	0.505	0.72	0.243	0.465	0.452	0.398	−0.0146	0.00998	0.0481	0.037
BTH 2 h	0.519	0.475	0.371	0.342	0.583	0.379	0.413	0.285	0.348	0.215	0.0799	0.203
BTH 5 h	0.535	0.568	0.536	0.593	0.526	0.517	0.54	0.366	0.124	0.113	0.0212	0.104
BTH 10 h	0.508	0.591	0.615	0.589	0.48	0.532	0.622	0.335	0.147	0.087	0.0905	0.0869
BTH 24 h	0.226	0.261	0.283	0.507	0.387	0.431	0.478	0.512	0.152	0.0848	0.066	0.31
BABA 2 h	0.522	0.429	0.459	0.471	0.605	0.558	0.548	0.253	0.128	0.11	0.0336	−0.00699
BABA 5 h	0.343	0.388	0.52	0.558	0.389	0.571	0.506	0.186	−0.021	0.000219	0.0123	−0.0925
BABA 10 h	0.399	0.447	0.597	0.609	0.383	0.537	0.596	0.23	0.0205	0.0221	0.0512	−0.0859
BABA 24 h	0.278	0.322	0.419	0.575	0.263	0.497	0.446	0.313	−0.000639	0.00155	0.0748	0.0636

those of SA-treatment, whereas they were less similar to the defense-induction profiles after MeJA/ethephon treatment. In the Col-0 plants treated with BTH, 134 genes showed increased expression with a peak in overall mRNA production at 5 h. The results of the cDNA microarray analyses indicated that BTH activates plant defense responses via the SA dependent signaling pathway, and to a lesser extent via the ET- and JA-dependent pathway (Figure 2C). Then, we compared the BABA microarray data set (220 genes) with that of SA, MeJA and ethephon. Treatment of Col-0 plants with BABA increased the expression of *PR-1* and *PR-4* genes, which are involved in the SA- and JA/ET-signaling pathways respectively. However, the expression profiles of stress-related genes after BABA treatment were generally more similar to those of SA treatment than those of MeJA/ethephon treatment (Figure 2D). It is also known that BABA-treated plants accumulate products associated with disease resistance including phenolics, callose, PR-proteins, salicylic acid, and hydrogen peroxide (Cohen 2002). Hence, BABA triggers a primed state in the plant that enables a more efficient activation of SA-, JA- and ET-dependent defense mechanisms. As mentioned above, the classification and characterization by the patterns of gene expression is more accurate and useful than by the correlation coefficient between each experiment pair.

The broad-spectrum activity of BTH compounds has been reported to protect dicotyledonous and monocotyledonous plant species against a number of bacterial, fungal and viral diseases, suggesting an indirect mode of action via activation of plant defense mechanisms (Friedrich et al. 1996; Görlach et al. 1996; Lawton et al. 1996; Benhamou and Bélanger 1998; Narusaka et al. 1999; Cole 1999; Godard et al. 1999; Buonaurio et al. 2002; Babu et al. 2003). Similar to BTH, BABA is also known to induce resistance against a broad spectrum of pathogens in many plant species, containing the *Arabidopsis* plant (Cohen 2002; Jakab et al. 2001). Therefore, a model screening system for candidate plant activators in *Arabidopsis* is a useful tool. BABA protects potato and *Arabidopsis* from infection with *Phytophthora* species but not BTH (Si-Ammour et al. 2003). In addition, many potato cultivars have high endogenous SA levels (Coquoz et al. 1995). Therefore, it seems that the resistance of potato against pathogens is different from that of *Arabidopsis*. In several cases, screening and assessing systems except *Arabidopsis* are also necessary to identify the novel active candidate for plant activators.

In summary, we assessed known plant activators by initially using a *GUS* reporter gene system in which the expression of *GUS* was driven by the promoter of either of two selected defense-associated genes. Subsequently, microarray analyses validated the strategy. From the data gathered in this study, we have identified molecular phenotypes of plant activators, independently of visible phenotypes. These plant activators were then classified according to the patterns of gene expression that they induced and the signaling pathway to which each plant activator belongs was revealed. The knowledge gained here will enable the development of new plant activators and will offer novel perspectives for engineering durable resistance in crop plants. We used cDNA microarrays to assess how plant activators affect the expression of stress related genes in *Arabidopsis* plants. Using these data, we will link bioinformatics with chemoinformatics by correlating gene expression profiles with the activity of plant activators. This is the first report of integration of large databases on gene expression and molecular pharmacology for agrichemicals.

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