

Evaluation of natural and synthetic stimulants of plant immunity by microarray technology

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Summary

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- Induction of local defence, as well as systemic resistance, of plants is associated with transcriptional reprogramming. Here we report on defence gene induction by natural and synthetic stimulants of plant immunity.
- Gene expression changes in *Arabidopsis thaliana* were monitored in response to several plant immunity stimulants (plant activators) using Northern blotting and an application-based array representing c. 750 genes involved in several aspects of plant defence and/or plant stress.
- The commercial plant activators Bio-S, Neudo-Vital and PRORADIX have been shown to induce systemic resistance. Here, Neudo-Vital, PRORADIX and Bio-S treatment induced different patterns of salicylic acid (SA) and jasmonic acid (JA) accumulation. Gene induction by these plant activators proved to be very complex. Rather than simply mimicking one of the known defence pathways induced by SA or JA, the response to the plant activators showed aspects of both major defence systems. A general feature was the transient activation of JA biosynthesis genes, combined with a much more sustained SA-associated defence gene induction.
- Our results demonstrate that plant immunity stimulants activate systemic immunity at the transcriptional level, and they provide insight into the coordinated transcriptional regulation of several classes of plant defence genes.

Key words: DNA array, jasmonic acid (JA), plant activators, plant defence, plant immunity, plant strengtheners, salicylic acid (SA).

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Introduction

Active disease resistance in plants depends on the plant's ability to recognize a pathogen and initiate a defence response. If such recognition occurs, a large number of genes are activated and a systemic response is triggered (Rushton & Somssich, 1998). Systemic resistance may result from induced tissues being primed to provide a rapid and strong defence response following subsequent pathogen challenge (Conrath *et al.*, 2002). One of the best-studied examples of these induced defence responses is systemic acquired resistance (SAR). This is mediated via a salicylic acid (SA)-dependant pathway (Durner *et al.*, 1997). It results in increased resistance to a broad spectrum of pathogens throughout a plant in response to localized necrosis caused by pathogen infection. Another

systemic resistance is mediated via jasmonic acid (JA) and ethylene-induced systemic resistance (ISR) (Pieterse & van Loon, 1999). Plant resistance is associated with activated expression of a large number of defence genes whose products may play important roles in the restriction of pathogen growth and spreading. Typical marker genes of SAR are for example encoding pathogenesis-related protein PR1 or other acidic PR genes while jasmonic acid induces proteinase inhibitors, defensins, thionines and basic PR genes (Zhou, 1999). Resistance mediated through SA and JA is thought to act antagonistic (Dong, 2001; Thaler *et al.*, 2002; Traw *et al.*, 2003).

The utilization of the natural plant immunity response has long been a goal of modern biodynamic agriculture. Modern biotechnology has developed well-defined components that induce systemic resistance. One well characterized example is

S-methyl benzo[1,2,3]thiadiazole-7-carbothioate (benzothiadiazole). It is a chemical analogue of SA, and as such, benzothiadiazole induces resistance through the SA-dependent pathway (Achuo *et al.*, 2002). Novartis/Syngenta introduced the compound as disease control component under the trade name BION (Kunz *et al.*, 1997). The application of BION induces expression of a similar pattern of defence transcripts as application of SA (Friedrich *et al.*, 1996; Lawton *et al.*, 1996). Within 4–12 h after application a strong induction of PR genes encoding class III chitinases, basic glucanases and acidic peroxidases was detected. The activation was long-lasting and no significant reduction of this overexpression of the PR genes could be found even 20 d after initial application (Friedrich *et al.*, 1996).

To date, several other projects have examined gene induction by defined plant activators. Another commercially available product, Oxycom (Redox Chemicals Inc., Burley, ID, USA), which contains reactive oxygen species (ROS)-generating systems, SA and other chemicals with potential fertilizer action was also shown to induce SAR- and PR-genes in tobacco (Yang *et al.*, 2002). The rice pesticide Probenazole (Oryzmate, Meijiiseika Co., Tokyo, Japan) was tested for its capacity to induce systemic resistance; it also induces genes from the SAR pathway. The induction appears to be upstream of SA induction as tested transgenic NahG plants do not show any disease induction and no expression of PR genes (Nakashita *et al.*, 2002a). Similar results were also found for plant activators based on chloroisonicotinamide (Nakashita *et al.*, 2002b). Another well-characterized substance is harpin. This elicitor from *Erwinia* and *Pseudomonas* species is the active ingredient in the plant activator Messenger (Eden Bioscience Co., Bothwell, WA, USA), which induces a hypersensitive response and eventually leads to SAR gene expression (Dong *et al.*, 1999; Krause & Durner, 2004). Interestingly, until now there has been no report on a plant activator applied in agriculture that induces resistance via the jasmonic acid pathway.

Unlike the well-defined plant activators, defence gene induction by natural and complex plant activators has never been analysed. While extracts of *Urtica dioica* or *Equisetum* spp. have long been used to increase resistance to different pests and pathogens, there are no data on gene induction associated with systemic resistance (Lust, 1979; Diver, 1998). Here we present data on the transcriptional response of *Arabidopsis thaliana* to three different plant activators with complex ingredients. We applied microarray technology which has become a useful

tool for the analysis of genome-scale gene expression, and which has been used to study the gene expression in *A. thaliana* in response to mechanical wounding, insect feeding and in response to fungal pathogen and signalling molecules.

Materials and Methods

Plant material

All experiments were done with *A. thaliana* cv. Columbia 0. The plants were grown in growth chambers at 18°C in the dark and 20°C in the light and 14-h light cycle. Experiments were conducted with plants 4–6 wk after germination. Plant material was harvested, frozen in liquid nitrogen and stored at –80°C. All experiments were done in three replicates and for each replicate and time point 10 individual plants were treated. All experimental values were determined after pooling of all plants and replicates. Appropriate RNA pooling has been reported to provide equivalent power and improve efficiency and cost-effectiveness for microarray experiments (Kendziorski *et al.*, 2003; Peng *et al.*, 2003). Our experimental design corresponds to previously published array studies on the influence of external stimuli or SAR-inducing treatments, such as pathogens, salt, ozone, hormones, UV-B and nitrate, which were based on pooled samples and technical replicates (Maleck *et al.*, 2000).

Biological plant activators

All test substances are listed in Table 1. BION was purchased from Novartis, Basel, Switzerland (now available from Syngenta), JA and SA from Sigma-Aldrich, Deisenhofen, Germany, and Neudo Vital from W. Neudorff GmbH KG, Emmerthal, Germany. Bio-S was kindly supplied by Gebrüder Schütte KG, Bad Waldsee, Germany; PRORADIX was kindly supplied by SOURCON-PADENA GmbH & Co. KG, Tübingen, Germany. All substances were dissolved in water and applied by spraying on the leaves.

DNA array

The *A. thaliana* DNA microarray currently being used for examining stress and/or redox-regulated gene expression involves longer fragments of synthetic or complementary DNA. Sequences are derived from databases, as polymerase chain reaction

Table 1 Plant defence activators

Name	Supplier	Active ingredient	Dosage
BION	Novartis/Syngenta	benzothiadiazole (BTH)	0.6 mM
Bio-S	Gebrüder Schütte KG, Bad Waldsee, Germany	Extract of several plant species	3%
Jasmonic Acid	Sigma-Aldrich, Deisenhofen, Germany	Jasmonic acid	1 mM
Neudo Vital	W. Neudorff GmbH KG, Emmerthal, Germany	Plant extract	0.2%
PRORADIX	Sourcon Padena GmbH & Co. KG, Tübingen, Germany	Ethanol extract of <i>Pseudomonas fluorescens</i> ssp. <i>Proradix</i>	5%

(PCR)-amplified partial open reading frames or specific 3' UTR sequences or are provided by others (Huang *et al.*, 2002). The array consists of *c.* 700 genes involved in or associated with plant defence and various cDNAs associated with primary metabolism and/or housekeeping. Specific 3' UTR sequences were used for members of the family of ABC-transporter, P450 monooxygenases, glucosyltransferases, glutathione-S-transferases and aquaporins (Glombitza *et al.*, 2004). Members of other gene families are represented by partial or complete coding sequence.

Microarray preparation A total of 200 μ l amino-modified PCR products were cleaned using 96-well multiscreen filter plates (Cat#MANU03050; Millipore, Bedford, MA, USA) and suspended in 20 μ l spotting solution (3 \times standard saline citrate (SSC) supplemented with 1.5 M betaine) and arrayed from 384-well microarray plates onto silylated microscope slides (CSS-100 silylated Slides; CEL Associates, Houston, TX, USA) using a DNA array robot (model GMS 417 from Genetic Microsystems). Printed arrays were incubated at room temperature over night and rinsed, twice in 0.1% sodium dodecyl sulphate (SDS) with vigorous agitation for 2 min, twice in double-distilled H₂O for 2 min, and once for 5 min in sodium borohydride solution (0.75 g NaBH₄ dissolved in 200 ml of PBS and 75 ml 100% ethanol). The arrays were submerged in H₂O for 2 min at 95°C, transferred quickly into 0.1% SDS for 1 min, rinsed twice in double-distilled H₂O, air dried and stored in the dark at room temperature.

Fluorescent probes Target RNA from NOR-3-treated *A. thaliana* cells was extracted using the TRIzol reagent according to the supplier's instructions (Gibco BRL, Div. of Invitrogen, Gaithersburg, MD, USA). Probes were made using indirect aminoallyl labelling method (described at <http://www.tigr.org/tdb/microarray/protocols.shtml>). Each mRNA sample (one control and one treated sample) was reverse-transcribed in the presence of Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech, Freiburg, Germany), and purified according standard protocols.

Hybridization and scanning Following reverse transcription, labelling and purification steps, Cy3- and Cy5-labelled probes were combined, 1 μ l salmon sperm DNA (20 μ g μ l⁻¹) and 1 μ l Poly(A)-DNA (20 μ g μ l⁻¹) were added, dried in a Speedvac (UniVac 150H, UniEquip, Munich, Germany) and dissolved in 50 μ l hybridization buffer (50% formamide, 6 \times SSC, 0.5% SDS, 5 \times Denhardt's). The probes were heated at 95°C for 3 min for denaturation and cooled on ice. The slides were immersed in prehybridization buffer (6 \times SSC, 0.5% SDS, 1% bovine serum albumin (BSA) and 1 μ g ml⁻¹ single stranded (ss) DNA) at 42°C for 45 min, thoroughly washed with double-distilled H₂O, and then air dried. Probes were hybridized to 1.0 cm² microarrays in 14 \times 14 Abgene geneframes in hybridization chambers (Geneworx AG Zinsser Analytic GmbH, Biorobotics Genemachines and Investigator Brand

Products, Oberhaching, Germany) overnight. Subsequently, the arrays were washed for 10 min at low stringency (2 \times SSC, 0.1% SDS), then for 5 min in 1 \times SSC, 0.1% SDS, and finally for 5 min at high stringency (wash buffer 0.1 \times SSC, 0.1% SDS). After a brief wash in double-distilled H₂O, the arrays were air dried and scanned using an AXON GenePix 4000 scanner (Axon Instruments, Union City, CA, USA). Separate images were acquired for each fluorophore at a resolution of 10 μ m per pixel. To identify differentially expressed genes we used the GENEPIX Pro 4.1 software. Background fluorescence was calculated as the median fluorescence signal of nontarget pixels around each gene spot. Less than 50% difference between background and signal resulted in elimination of the corresponding spot. For statistical and cluster analyses we used the ACUTY 3.1 software suite (Axon Instruments).

Northern hybridization

RNA from *A. thaliana* cells was extracted using the TRIzol reagent according to the supplier's instructions (Gibco/BRL). Northern analysis followed standard protocols. The *A. thaliana* sequences used as probes for hybridizations (Huang *et al.*, 2004) were labelled with digoxigenin as recommended by the manufacturer (Boehringer, Mannheim). Northern blots were done at least in triplicate. Typical expression profiles are shown.

Determination of SA and JA

Determination of SA and JA was done as replicates of three independent experiments. Extraction and quantification of free and conjugated SA was performed basically according to Meuwly & Métraux, (1993). Salicylic acid was detected by using a Shimadzu RF 535 fluorescence detector (Shimadzu, Duisburg, Germany) at excitation and emission wavelengths of 305 nm and 407 nm, respectively.

For JA determinations, plant tissue was shock-frozen with liquid nitrogen and processed as described by Mueller & Brodschelm (1994). For quantification, 100 ng of 9,10-dihydro-JA was added to the frozen cells prior to processing. Jasmonates were analysed as pentafluorobenzyl (PFB) ester derivatives by gas chromatography (GC)–mass spectrometry (SSQ quadrupole instrument; Finnigan, San Jose, CA, USA) operated in the negative ion chemical ionization mode using isobutane as reactant gas. [Molecular anions-PFB]-ions of JA-PFB (m/z = 209) and Dihydro-JA-PFB (m/z = 211) were monitored and JA levels were calculated from the GC peak areas of the selected ions.

Results

Evaluation of gene expression after treatment with biological plant activators via Northern blots

Increased resistance to pathogenic fungi can be assigned to a specific activation of defence systems, or to a better

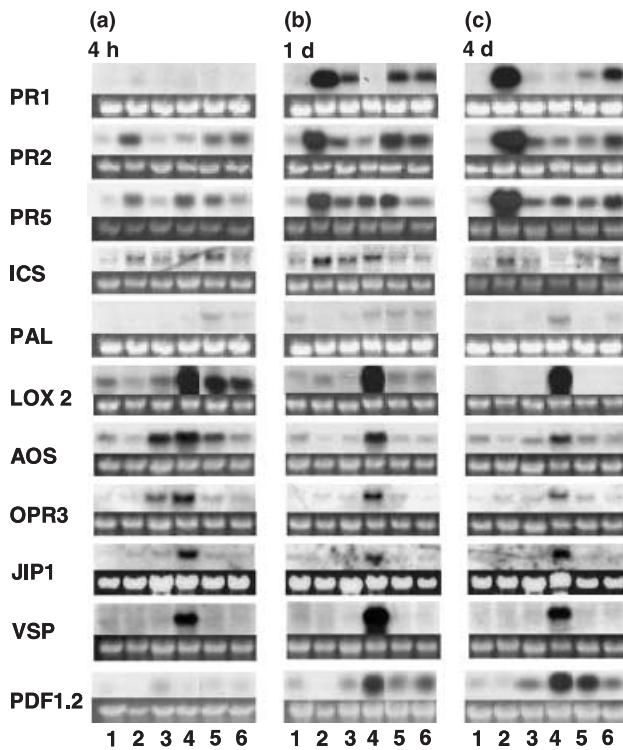


Fig. 1 RNA gel blot analyses after treatment with different plant activators. *Arabidopsis thaliana* plants were treated with plant activators (1, Control; 2, BION; 3, Bio-S; 4, JA (jasmonic acid); 5, Neudo-Vital; 6, PRORADIX) and total RNA was isolated at 4 h (a), 1 d (b) and 4 d (c) after treatment. Ten micrograms of total RNA was separated on formaldehyde agarose gels and subsequently transferred to nitrocellulose membranes. Digoxigenin-labelled DNA fragments were used for detection.

overall constitution of the plant. A general increase in plant constitution (for example via fertilization) can lead to healthier plants. To verify that the increased resistance of the stimulated plants did originate from the induction of resistance genes we tested the plant activators by Northern blot analysis. These analyses included marker genes of the SAR pathway (*PR1*, *PR2* and *PR5*), the ISR/JA pathway (*VSP*, *JIP1* and *PDF1.2*), JA-biosynthesis (*LOX2*, *AOS* and *OPR3*) and the biosynthetic genes *PAL* and *ICS1*. The results of this experiment are shown in Fig. 1. The positive controls show clear induction of the corresponding genes: in JA-treated plants there is a prominent band showing the induction of *PDF1.2* after 24 h. In the BION-treated plants a strong induction of *PR1*, *PR2* and *PR5* can be found after 1 d. Control plants show no or only very basal expression of PR-genes or JA-responsive genes such as *PDF1.2*. The plant activator treated samples also show the activation of several defence genes (Fig. 1): *PR1*, *PR2* and *PR5* are induced after treatment with Bio-S, Neudo-Vital and PRORADIX. *PDF1.2* is significantly induced 4 d after treatment with Neudo-Vital but there also is a slight induction after PRORADIX and Bio-S treatment. All three plant activators tested show a transient induction of JA-synthesis

genes after 4 h but in different manners. While Proradix-application only increased the expression of *LOX*, Neudo-Vital induced the expression of *LOX* as well as *AOS*. Only Bio-S induced the expression of *LOX*, *AOS* and *OPR3* thus activating the complete octadecanoid pathway leading to JA synthesis. The induction of JA-synthesis genes was transient for all three plant activators and could not be found 24 h or later after application while the induction was clearly longer-lasting after application of JA (Fig. 1). Interestingly, unlike the JA-biosynthesis genes the JA-responsive genes *VSP* and *JIP1* were not induced – a situation reminiscent of the transient activation of JA-biosynthesis by nitric oxide (Huang *et al.*, 2004).

DNA array analysis of transcripts in *A. thaliana* in response to biological plant activator treatment

We first studied gene expression dynamics in plant activator-treated *A. thaliana* plants by using a custom-designed cDNA microarray that included *c.* 700 defence-related genes encoding PR-proteins or proteins induced by oxidative stress, cold, UV, ozone or heavy metals and 50 genes associated with primary metabolism. We defined induction or repression of a gene as a minimum twofold change in its transcript level. Array hybridizations were based on four replicates and a dye-swap. We applied the following selection procedure to our expression data: (1) only signals more than twofold above local background level were considered, (2) only expression ratios higher than 2.0 were regarded as significant and (3) values with CV-values over 10 were emitted. These very rigorous criteria implicate that our procedure ignores such genes with relatively low basal expression ratios (see also the Supplementary Material, Table 2).

While the array hybridizations included several technical replicates and controls (e.g. dye-swap), we had to pool the biological samples. Appropriately designed RNA sample pooling can improve efficiency and cost-effectiveness, for many types of microarray experiments when inferences are made at the group level (Kendzierski *et al.*, 2003; Peng *et al.*, 2003). This type of analyses has frequently been applied for plants. The response of plants to numerous external stimuli such as pathogens, salt, ozone, hormones, ultraviolet (UV)-B radiation and nitrate has been analysed with pooled samples and technical replicates (Maleck *et al.*, 2000). However, RNA pooling may also have adverse consequences, and can be inappropriate in some cases. In plants, analyses of mutant screens, knockout-transcriptomes or organ-specific/developmental processes should be analysed on a basis of several biological repeats (Jiao *et al.*, 2003). An uncontrolled variable affecting a single plant and inducing the expression of one or more genes from a low to high level would be detected in the pooled sample and the data recorded as either treatment-induced (if the variable affected a plant in the treated pool) or treatment-repressed (if the variable affected a plant in the untreated pool). Pooling will also prevent later analysis of our data on variables that

Table 2 Short summary of DNA array analysis of transcripts in *Arabidopsis thaliana* in response to biological plant activator treatment

ID	Name	Pathway	BION			Bio-S			JA			Neudo-Vital			PRORADIX		
			4 h	1 d	4 d	4 h	1 d	4 d	4 h	1 d	4 d	4 h	1 d	4 d	4 h	1 d	4 d
At2g14610	PR1	SAR	0.16	40.7	52.1	0.62	27.8	6.76	0.97	0.88		1.39	18.6	3.49	1.06	3.99	16.3
At1g75040	PR5	SAR	0.2	14.9	20.5	1.91	5.44	5.31	0.96	0.7	0.63	2.2	3.64	1.19	1.3	2.48	5.47
At3g57260	PR2	SAR	0.53	7.45	13.2	1.57	2.22	2.09	1.78	1.18	1.53	1.23	2.64	1.31	0.7	1.82	2.96
At3g04720	Pre-hevein-like	SAR	0.2	3.75	3.33	6.23	1.71	1.44	2.03	1.34	1.38	5.57	2.92	1.51	5.56	1.66	2.07
At3g12500	Basic chitinase	SAR	0.62	3.27	3.37	0.81	1.34	1.07	0.94	0.82	1.05	0.96	4.95	1.61	1.22	1.65	2.48
At3g49120	Peroxidase	SAR	0.39	3.21	5.27	2.44	2.68	3.35	1.96	2.62	1.9	2.77	3.45	2.06	1.53	1.33	3.12
At1g64280	NPR1	SAR	0.39	2.48	2.12	1.48	1.41	1.26	0.77	1.15	0.92	1.38	1.21	0.94	1.28	0.85	1.29
At1g55020	LOX1	JA	0.89	1.55	1.31	3.36	1.31	1.24	4.12	4.85	4.15	1.27	1.17	1.3	0.75	1.09	1.46
At3g45140	LOX2	JA	1.02	1.01	0.87	3.64	1.13	1.34	4.45	6.23	4.29	1.21	0.95	1.06	0.55	0.7	1.01
TC103526	SAP	JA	0.39	1.36	1.35	1.62	1.15	1.03	4.63			1.37	1.3	1.2	1.06	0.78	1.61
At1g72260	Thionin	JA	0.93	1.36	1.05	0.63	0.84	0.75	1.22	1.15	2.23	1.35	1.19	1.05	1.26	1.6	0.99
At3g16470	Jacalin lectin	JA	0.75	1.3	1.2	3.26	1.41	1.42	11.7	12	7.96	1.9	1.65	1.36	0.91	0.94	0.74
At3g16420	MBP	JA	0.99	0.88	1.05	1.56	1.3	1.25	5.87	3.37	2.05	1.83			0.76	0.86	0.7
At5g42650	AOS	JA	1.24	0.62	0.71	5.39	0.93	0.96	6.78	5.68	2.83	1.12	0.7	0.93	0.6	0.82	0.89
At5g24780	VSP	JA	2	0.6	0.67	0.99	1.33	1.13	15	123	96.4	0.63	0.61	1.16	0.56	1.24	1.17
At5g44420	PDF1.2	JA	2.14	0.42	0.68	0.88	1.16	1.81	1.15	2.18	3.21	1.23	1.67	2.42		1.08	1.31
At1g02930	GST3	detox	0.13	8.02	5.36	16.3	3.04	1.79	2.34	1.46	1.25	7.18	3.68	1.66	6.59	2.57	3.5
At1g02920	GST11	detox	0.09	7.09	6.37	11.1	2.54	2.64	2.31	1.65	1.19	9.07	3.91	1.5	6.1	2.23	3.17
At2g02930	GST16	detox	0.11	6.65	5.04	3.44	4.39	3.07	1.4	0.57	0.72	4.53	2.71	1.46	2.25	1.91	1.8
At2g30140	GT-51	detox	0.1	4.24	3.97	3.06	2.02	1.86	4.04	1.03		5.57	1.76		2.89		0.99
At1g04120	ABC-O	detox	0.15	4.2	3.39		0.87	1.03	1.24	1.89	3.94	2.68	0.91	1.17	1.73	1.32	1.48
At4g02520	GST2	detox	0.29	2.79	2.51	2.41	2.04	3.68	1.11	0.68	0.81	3.56	2.34	1.25	1.93	1.37	1.63
At1g59870	ABC-Transporter	detox	0.6	2.72	1.79	1.88	2.19	2.3	1.26	1.95	3.32	1.62	1.59	2.25	2.25	1.32	2.63
At1g30400	ABC-Transporter	detox	0.27	2.35	2.01	1.04	0.98	1.06	1.44	0.95	1.37	3.14	1.25	0.67	1.43	0.95	0.64
At2g30870	GST4	detox	0.37	1.85	1.96	3.19	1.1	1.9	4.51	1.94	1.46	3.84	1.69	1.41	1.66	0.95	2.03
At1g25220	Anthranilate synt.	SM	0.48	1.22	1.38	3.39	1.31	1.31	2.23	1.27	1.35	2.31	1.39	1.19	2.21	1	1.32
At5g05730	Anthranilate synt.	SM	1.2	0.8	0.86	1.29	1.33	1.15	10.6	78.8	65.3	1.44		1.1	0.73	0.96	1.27
At5g54810	Tryptophane synt.	SM	0.82	0.93	1.43	3.98	1.15	1.17	1.96	1.86	1.87	2	1.47	1.47	1.5	1.22	1.34
At3g54640	Tryptophane synt.	SM	3.17	0.68	0.42	0.22	0.65	0.67	1.03	0.74	1.29	0.62	1.36	1.36	3.52	2.78	1.44
TC115647	Chalcone isom.	SM	2.73	0.61	0.47	0.44	0.81	0.51	0.9	0.65	1.35	0.63	1.42	1.46	1.75	2.15	1.01
At3g55120	Chalcone isom.	SM	3.46	0.59	0.39	0.21	0.64	0.61	1.03	0.72	1.1	0.52	1.37	1.27	3.41	2.78	1.27
At5g13930	Chalcone synt.	SM	2.79	0.52	0.58	0.65	0.96	1.08	0.48	0.96	0.8	0.29	0.63	0.84	0.57	1.1	1.35
At1g21250	Serine/threonine kin.	PI	0.14	5.28	2.85	2.62	4.03	1.71	1.63	1	1.3	2.6	1.87	1.1	3.27	1.32	1.18
At5g06870	PGIP2	PI	0.3	3.91	3.11	4.76	1.45	1.39	2.74	2.55	1.49	4.34	2.37	1.48	3.48	1.64	2.42
TC104379	Aldose epimerase	PI	0.7	1.08	1.33	3.51	1.4	1.92	9.02	12.8	12	1.66	0.84	0.76	0.54	0.74	0.62
At5g20230	Blue copper binding	MS	0.23	6.99	8.56	4.21	1.52	1.62	3.49	2.42	1.35	5.26	5.07	2.06	4.89	2.62	3.63
At4g12480	pEARL1	MS	0.71	1.02	1.74	5.13	15.3	9.86	0.76	1.08	1.17	4.92	9.36	3.58	0.78	2.15	4.03
At1g20620	Catalase	OS	1.14	0.96	2.11	0.98	1.56	2.51	0.89	1.48	2.81	0.83	0.9	1.72	0.59	0.96	1.35
At3g10920	Putative [Mn] SOD	OS	1.23	0.75	0.82	2.06	0.92	1.01	2.69	2.48	1.36	0.85	0.71	0.86	0.57	0.86	0.94
H76549	HSP	HS	0.74	1.43	0.76	2.25	0.78	0.59	1.08	0.67	1.22	1.74	0.91	0.91	2.82	1.24	0.31
At3g15210	ERF4	TF	1.74	0.93	0.68	0.35	0.69	0.59	0.99	0.86	1.24	0.59	1.2	1.15	1.74	2.11	1.12
T44718	Sulfate transporter	M	2.57	0.69	0.5	0.26	0.73	0.66	0.94	0.73	1.25	0.54	1.33	1.12	2.22	2.29	0.99
TC103532	Seed imbibition	M	2.82	0.69	0.44	0.37	0.69	0.7	1.03	0.71	1.22	0.49	1.33	1.35	2.92	2.94	1.23
T20553	DNA repair	M	2.04	0.67	0.49	0.32	0.74	0.65	0.99	0.8	1.14	0.53	1.2	1.26	2.51	1.5	1.31

Changes in expression are highlighted: yellow, 2–2.5-fold activation; orange, 2.5–3-fold activation; red, more than threefold activation; mint-green, 2–2.5-fold repression; light green, 2.5–3-fold repression; dark green, more than threefold repression. HS, heat shock; OS, oxidative stress; MS, metal stress; PI, pathogen induced TF, Transcription factor, SM, secondary metabolism; M, Miscellaneous.

may have been ignored initially (slightly different age of our plants, growth conditions). Similarly, pooling will prevent users from finding differences in expression that might divide only one set of samples. However, since we took samples at various time-points and since several genes showed increased expression in two or more post-treatment pools (in response to two or more plant activators) it is likely that these genes were induced by the treatment. Thus, we think a pooling approach is justified as long as such data are verified by additional methods such as Northern blotting and independent RNA (as shown in Fig. 1). Nevertheless, further investigations should be much more detailed and should consider more experimental factors.

The temporal transcription program of was analysed 4 h, 1 d and 4 d after treatment of plants with Bio-S, PRORADIX

and Neudo-Vital, respectively. Induction of several enzymes involved in resistance could be observed after treatment with both biological plant activators. Analyses of these data revealed that 281 genes showed significant differential expression in response to one or more of the treatments. The strongest overall response was after BION treatment. After treatment, a strong induction of *PRI*, *PR2* and *PR5* could be observed. We categorized the responsive genes into several major groups. While *PR1* is a reliable marker of the SAR pathway, glucosyltransferases are often expressed as part of a general detoxification process. Others, such as blue copper-binding protein (Ezaki *et al.*, 2001) and pEARL1 are reported as metal-stress induced. Overall, the results from our Northern analyses (Fig. 1) correspond well with the data from our microarray analyses. Table 2 gives a short summary of some

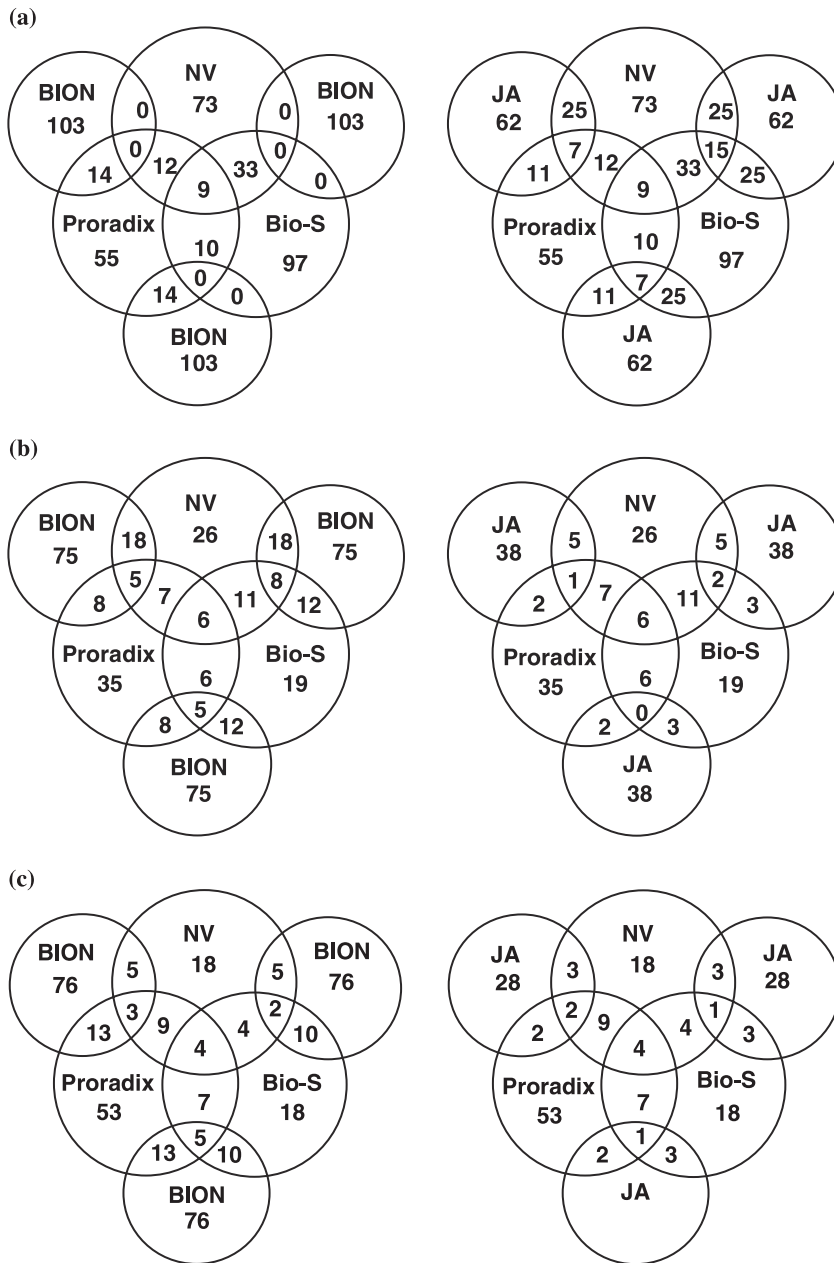


Fig. 2 Venn diagrams of genes induced by various plant activators. *Arabidopsis thaliana* plants were treated with plant activators and total RNA was isolated at 4 h (a), 1 d (b) and 4 d (c) after treatment. Array hybridizations were as described. The Venn diagrams show the number of overlapping and nonoverlapping regulated genes with ratios of > 2 or < 0.5 after treatment with plant activators. For the sake of clarity, the comparison of the plant activators with JA (jasmonic acid) and BION is shown in separate diagrams. Data are based on pooled RNAs of three independent experiments (10 treated plants each).

interesting genes induced by plant activators. Complete sets of array data can be found in the Supplementary Material.

The Venn diagram test supports comparison of high/low-expressed gene sets between treatments with different plant activators. Regions of overlap in the diagram represent genes that are conditionally coregulated with the respective groups of genes. Here, we compare gene expression induced by plant activators with BION- and JA-treatment, respectively. Bio-S, Neudo-Vital and PRORADIX did not show a typical fingerprint of either SA- or JA-associated responses. Instead, they induced genes from several pathways. Four hours after plant activator treatment a number of induced genes were also

expressed in JA-treated plants. Bio-S especially shows a high level of coexpression with JA (e.g. the JA-biosynthesis genes lipoxygenase (LOX) and allene oxide synthase(AOS)) at that time-point, while there is no concurrence to BION-treated plants. With Bio-S, 26% of the regulated genes show the same reaction as with JA; for Neudo-Vital this value can be 34%. PRORADIX shows coexpression with BION as well as with JA (Fig. 2). Strikingly, after 24 h the transcriptional profile had changed and Bio-S as well as Neudo-Vital show higher coregulation with BION-treated plants. The change of expression patterns becomes also visible by cluster analysis (Fig. 3), a hierarchical tool that groups coregulated genes by

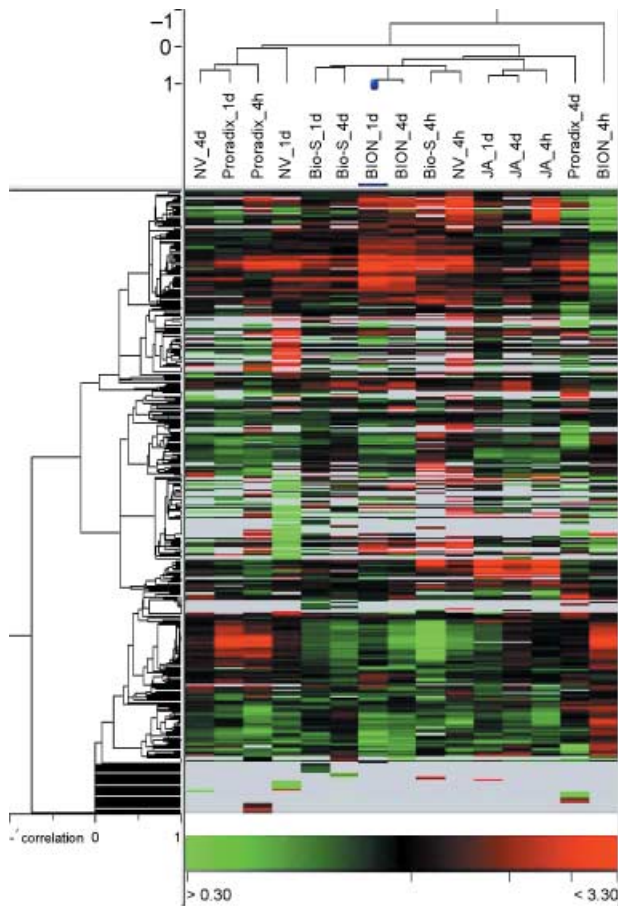


Fig. 3 Cluster analysis of plant (*Arabidopsis thaliana*) transcripts after treatments with different plant activators. The steady-state transcript levels corresponding to genes on our microarray (see experimental procedures) were analysed 4 h, 1 d and 4 d after plant activator treatment. The relative abundance of any one transcript in treated plants was compared with untreated control plants. Transcript ratios greater (smaller) than 1 are indicated by gradation of red (green) of increasing intensity. A transcript was considered to be enriched (depleted) when ratio was > 2 (< 0.5). Data represents four replicated array spots from the pooled RNAs of three independent experiments with 10 plants each. The figure was generated using the programs GENE PIX PRO 4.1 and ACUITY 3.1 (Axon Instruments). NV, Neudo-Vital.

both related regulation patterns and expression amplitudes: one branch represents the different time-points of JA treatment and includes Bio-S and Neudo-Vital 4 h after treatment. Later time-points of Neudo-Vital treatment are clustered together with PRORADIX, and with Bio-S are in a separate branch. The 4-h BION treatment is not clustered.

Cluster structure of gene expression data obtained from DNA microarrays is often analysed and visualized with the Self-Organizing Map (SOM) algorithm. The SOM forms a nonlinear mapping of the data to a two-dimensional map grid that can be used as an exploratory data analysis tool for generating hypotheses on the relationships (cotranscription) of genes. Nine SOMs with different expression profiles were determined (Fig. 4). Self-Organizing Map c9 includes the

robust SAR marker *PR-1* and closer analysis of this cluster revealed that other PR proteins that have reported functions in SAR are also clustered in SOM c9 (see Supplementary Material). This is consistent with previous SAR transcriptome analysis (Maleck *et al.*, 2000). Genes from this cluster are not upregulated after JA-treatment but show an increased expression after Neudo-Vital and PRORADIX treatment. Several genes involved in JA and ethylene signalling are clustered together in SOM c7. These genes are included in the octadecanoid pathway that leads to JA biosynthesis (*AOS*, *LOX*) (Huang *et al.*, 2004), formation of ethylene receptors (*ETR1*, *EIN4*) or upstream region of the JA-responsive genes (*VSP1*) (Seo *et al.*, 2001; Ellis *et al.*, 2002). This group of genes was upregulated after JA treatment, but also shortly after Bio-S and Neudo-Vital treatment. While their expression was prolonged after JA treatment the induction after Bio-S and Neudo-Vital was rather transient. *PDF1.2*, another marker gene for JA (Brown *et al.*, 2003) was not included in this cluster. This gene is regulated much later than the genes represented in SOM c7. *PDF1.2* is clustered into SOM c2 which includes stress genes that are upregulated 4 d after JA, Neudo-Vital and PRORADIX treatment.

Influence of plant activators on the defence signals salicylic acid and jasmonic acid

Systemic resistance (SAR and/or ISR) is usually mediated via SA or JA. This makes these plant hormones excellent markers for systemic resistance. Therefore, the induction of SA and JA by plant activators was tested (Figs 5 and 6).

The highest induction of free SA was found after treatment with BION and highest levels of conjugated forms of SA after BION, Neudo-Vital and PRORADIX-treatment (Fig. 5). It should be noted that most of the SA was in the conjugated form, with free SA sometimes comprising less than 10% of total SA. Similar results have been reported for tobacco resisting pathogen attack or after treatment with elicitors.

Jasmonates (including JA) help in regulating plant growth and development and they appear to participate in leaf senescence and in the defence mechanism against fungi. In many cases, SA and JA work in an antagonistic manner. Here, enhanced levels of JA were observed as early as 1 d after application of the various plant activators, with the highest induction after treatment with Bio-S (Fig. 6). This is in accordance with our finding that only Bio-S shows enhanced expression of all the tested genes of JA synthesis, namely *LOX*, *AOS* and *OPR3* (Fig. 1). PRORADIX and Neudo-Vital show no enhanced expression of *OPR3*, thus lack the last step in JA synthesis.

Discussion

Defence gene activation by plant activators has been reported for BION (PR-genes in wheat, tobacco and *A. thaliana*;

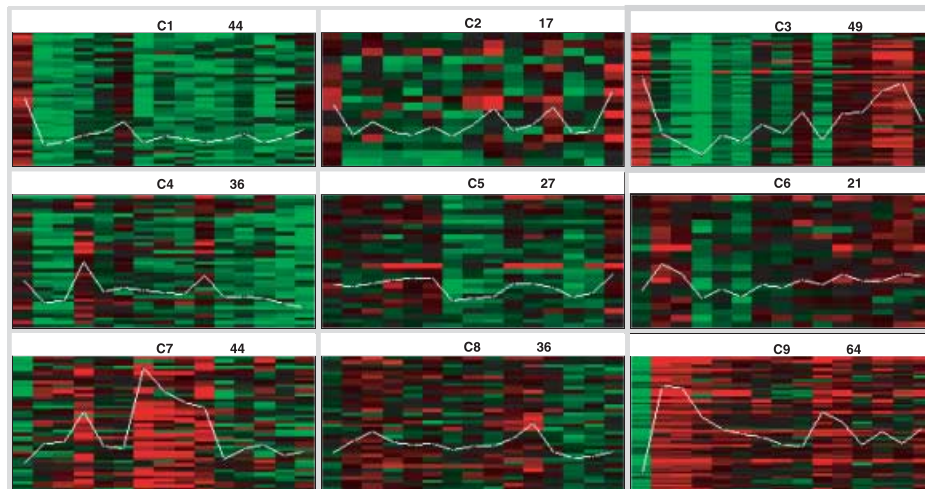


Fig. 4 Self-organizing map clusters of expression profiles. The plots are based on gene expression data shown by Figs 2 and 3. Each graph displays the mean expression pattern of the genes in that self-organizing map (SOM) cluster. The number of genes in each cluster is at the top right of each SOM. The figure was generated using the programs GENE PIX PRO 4.1 and ACUTY 3.1 (Axon Instruments).

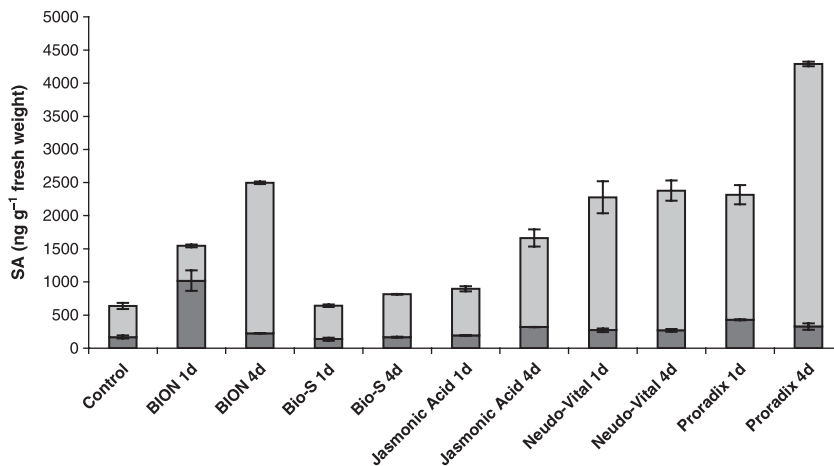


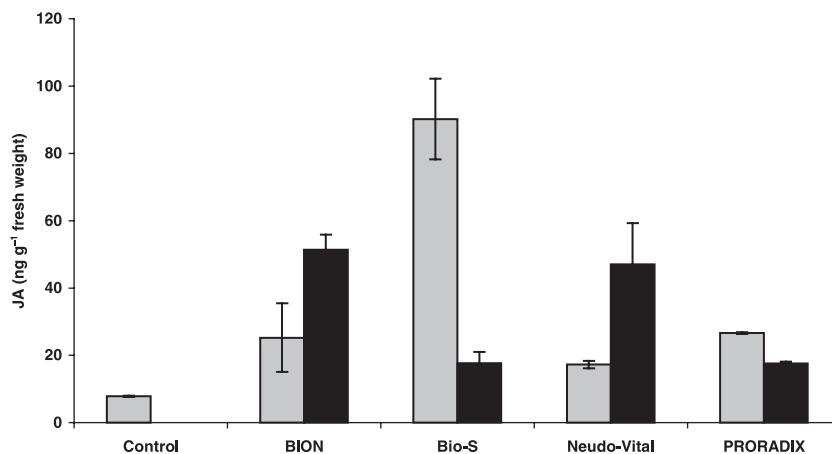
Fig. 5 Effects of plant activator treatment on salicylic acid (SA) concentrations in *Arabidopsis thaliana* leaves. Plant activator treatment was as described. Three experiments were analysed in duplicate, respectively (means are shown). Sampling was 1 d and 4 d after treatment. Light tint, conjugated; dark tint, free.

Görlach *et al.*, 1996), Milsana (chalcone synthase and isomerase in cucumber; Fofana *et al.*, 2002), Oxycom (PR-1a, 1g and 3a in tobacco; Yang *et al.*, 2002) and Probenazole (several PR-genes in tobacco; Nakashita *et al.*, 2002a). However, most of these studies focused on very few marker genes of induced plant defence. So far, no detailed study on the effect of plant activators on gene expression has been published. Microarray technology has become a useful tool for the analysis of gene expression. cDNA or oligo sequences arrayed on a glass slide, at a density of up to 1000 genes cm⁻², are hybridized simultaneously to a two-colour fluorescently labelled cDNA probe pair prepared from RNA samples of different cell or tissue types, allowing direct and large-scale comparative analysis of gene expression. This technology, which uses expressed sequence tags (ESTs), has been used to study the gene expression in *A. thaliana* in response to mechanical wounding and insect feeding (Reymond *et al.*, 2000). Defence-signalling pathways (e.g. SAR) have been

analysed using fungal pathogen and signalling molecules (Maleck *et al.*, 2000; Schenk *et al.*, 2000; Cheong *et al.*, 2002).

The aim of this study was to obtain insight into the mode of action of different plant activators, which are believed to induce systemic resistance (SAR and ISR) in plants. We tested the expression of different marker genes by Northern analyses and used a custom-designed microarray. *PR1* is a widely used marker gene for SAR while *PDF1.2* is a marker for JA-dependent responses (and possibly ISR). The positive control for *PDF1.2* was JA; positive control for *PR1* was BION (a SA substitute). Interestingly, all three plant activators (Bio-S, Neudo-Vital and PRORADIX) did induce both SAR-type genes, which are activated via the SA pathway, but also ISR- or JA-dependent genes. This is interesting, as resistance mediated through these two key signal components is thought to act antagonistically (Dong, 2001; Thaler *et al.*, 2002; Traw *et al.*, 2003). However, there is more and more evidence that

Fig. 6 Effects of plant activator treatment on jasmonic acid (JA) concentrations in *Arabidopsis thaliana* leaves. Plant activator treatment was as described. Three experiments were analysed in duplicate, respectively (means are shown). Sampling was 1 d (light tinted columns) and 4 d (dark tinted columns) after treatment.



the major defence signalling pathways (SA and JA) do not function independently (Kunkel & Brooks, 2002). Previous microarray analyses point to a positive interaction between these two signal molecules and revealed a number of defence-genes that are coinduced by SA and JA (Schenk *et al.*, 2000). Moreover, after infestation with the aphid *Brevicoryne brassicae*, *A. thaliana* plants showed an induction of both *PR1* and *PDF1.2* (Moran *et al.*, 2002). In our tests we found no clear separation of the two pathways. Treatment with complex plant activators induced genes that are associated with SAR but also genes known to be induced by JA (Table 2). Like jasmonates, which are known to induce their own biosynthesis genes (Sasaki *et al.*, 2000, 2001), the application of, for example Bio-S induced *LOX*, *AOS* and *OPR3* and after 1 d the plants produced JA. Surprisingly these genes were downregulated after only 1 d and the JA levels returned to control levels after 4 d (Fig. 6). Thus, Bio-S and also Neudo-Vital – for which an induction of *LOX* and *AOS* was found (Fig. 1) – first induced a JA-like response, subsequently followed by a BION-like gene activation (Fig. 3). Transient expression of the genes of the octadecanoid pathway (*LOX*, *AOS* and *OPR3*) in *A. thaliana* has been reported after treatment with nitric oxide and might be part of a priming phenomenon (Huang *et al.*, 2004). Despite this strong activation of genes involved in JA biosynthesis the authors found no induction of typical JA-inducible genes. This again is in accordance with our findings that none of the plant activators led to an increase of transcript levels of *JIP* or *VSP* (Fig. 1), which are typical representatives of JA-inducible genes.

Treatment with the plant activator PRORADIX revealed much more similarities to BION treatment (i.e. SAR like response) than Bio-S and Neudo-Vital (Fig. 2). Not only in the amount of coexpressed genes but also in the fact that induction seems to increase further from 1 to 4 d after treatment. Sustained gene induction by BION has been reported for *A. thaliana* and wheat (Görlach *et al.*, 1996). Conversely, detailed analyses revealed profound differences between BION and PRORADIX. Only *c.* 25% of the genes signifi-

cantly regulated after PRORADIX treatment were influenced by BION treatment. A prominent example was the induction of *ERF4* after 24 h. Transcription factors of the *ERF* class are believed to integrate signals from ethylene and jasmonate pathways in plant defence responses (Lorenzo *et al.*, 2003). *ERF* transcription factors have two *ERF/AP2*-domains for DNA binding and belong to the group of GCC box binding factors. Five different types have been described in *A. thaliana* and they are key factors regulating responses to pathogens, ethylene, wounding, cold, salt stress, and drought (Turner *et al.*, 2002).

Glycosyltransferases (GTs) and glutathione-S-transferases (GSTs) as well as ABC-transporters were activated by all plant activators tested. These genes are often involved in cellular detoxification processes (Dixon *et al.*, 2002; Martinoia *et al.*, 2002). Several ABC-transporters have been associated with various host–pathogen interactions and induction by pathogenic *Alternaria brassicicola* has been reported (Campbell *et al.*, 2003).

Bio-S and especially Neudo-Vital induce the highest expression of PR genes after 1 d (Fig. 1). However, the strong expression of most of these genes diminished 4 d after the treatment. By contrast, BION and PRORADIX showed a much longer, sustained PR-gene induction. Four days after treatment with these plant activators, most PR-genes were at an even higher state of induction than immediately after the treatment. Such a sustained gene activation is assumed to be of central importance for a long-lasting resistance against pathogens (Sticher *et al.*, 1997). PRORADIX did induce a pronounced increase in SA levels that did not continue to rise after 1 d. Most of this SA is found in a conjugated form. As such, it is not active, but provides a pool readily available in case of infection (Hennig *et al.*, 1993; Chen *et al.*, 1995). Interestingly, our results show that after BION-treatment, there is an early increase of ACC (data not shown), followed by a strong induction of SA and subsequent induction of JA. New results show that lesion expansion is ethylene-dependent (Verberne *et al.*, 2003) and a model has been proposed, that

lesion formation is mediated via ethylene, lesion expansion via SA and lesion containment via JA (Langebartels & Kangasjärvi, 2004). All three hormones act together and are required for the optimal hypersensitive response. The same pattern has been found to a lesser extent in Neudo-Vital. Neudo-Vital is an extract of several different plant species and contains a mixture of fatty acids. Similar to BION, Neudo-Vital induces an early ACC peak (data not shown), an increase in SA after 1 d and a late JA peak. Overdosage of Neudo-Vital induced lesions and led to plant death after 2 d.

In most cases, establishment and maintenance of acquired resistance requires the participation of SA as a signal (Métraux & Durner, 2004). In addition to PR-gene induction (Fig. 1) plant activators activated SA-biosynthesis. This biosynthesis is mediated by two pathways: phenylalanine ammonia-lyase (PAL) is involved in synthesis of SA precursors and lignin biosynthesis (Mauch-Mani & Slusarenko, 1996). Accumulation of SA via PAL occurs in cells undergoing cell death at the site of infection (lesion formation). Surprisingly, microarray analysis did not reveal any significant induction of *PAL1* for any of the tested plant activators (Table 2; see the Supplementary Material). These results were confirmed by Northern analyses (Fig. 1). Only JA treatment yielded a moderate increase of PAL transcript after 4 d (induction factor 1.884). It is noteworthy that a second SA-biosynthesis pathway via isochlorismate synthase (*ICS1*) has been described for local and SAR resistance responses (Wildermuth *et al.*, 2001). As *ICS1* is not represented on our microarray we performed Northern blots to analyse *ICS1* transcripts (Fig. 1). Strikingly, these analyses revealed an elevation of *ICS* transcripts after treatment with all plant activators tested. This gene activation occurred as early as 4 h after BION and Neudo-Vital-treatment. Four days after treatment with BION and PRORADIX plants still show sustained, elevated levels of *ICS1*. These results match our findings that these two plant activators induce a prolonged increase of the amount of total SA (Fig. 5).

The mechanisms by which plant activators induce defence genes are poorly understood. Some genes clearly depend on the participation of endogenous signalling molecules, such as JA, SA or ethylene (Glazebrook, 2001). In some cases, plant activators might act as elicitors and cause HR-type resistance (Dong *et al.*, 1999), or they might act as or together with signalling molecules (Mercier *et al.*, 2001). At present, we do not know the molecular basis for gene activation by Bio-S, Neudo-Vital and PRORADIX. However, preliminary data show that these plant activators induce resistance against the fungi *Alternaria alternata*, *Botrytis cinerea* and *Cladosporium herbarum* (data not shown).

We conclude that this study has not only identified a large number of genes that are induced by treatment of *A. thaliana* with plant activators, but it might also provide new insights into interaction/overlap between the various types of induced and acquired defence. Unlike SA- or JA-mediated defence patterns, the action of Bio-S, Neudo-Vital and PRORADIX

is rather complex. The combination of defence genes induced by plant activators, together with the production of endogenous signalling molecules such as SA and JA may better represent the complex patterns found in true plant pathogen interactions than well-defined substances that trigger only certain pathways.

Supplementary Material

The following material is available as Supplementary material at <http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1211/NPH1211sm.htm>

Table S1 Supplement for Table 2. DNA array analysis of transcripts in *Arabidopsis thaliana* in response to biological plant activator treatment.

Table S2 Supplement for Fig. 4. Genes represented in the different SOM-clusters (SOM c1 – SOM c9) and their expression after plant activator treatment.

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