Phospholipase D Activation Is an Early Component of the Salicylic Acid Signaling Pathway in Arabidopsis Cell Suspensions[1][W][OA]

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Salicylic acid (SA) plays a central role in defense against pathogen attack, as well as in germination, flowering, senescence, and the acquisition of thermotolerance. In this report we investigate the involvement of phospholipase D (PLD) in the SA signaling pathway. In presence of exogenous primary alcohols, the production of phosphatidic acid by PLD is diverted toward the formation of phosphatidylalcohols through a reaction called transphosphatidylation. By in vivo metabolic phospholipid labeling with $^{33}P_i$, PLD activity was found to be induced 45 min after addition of SA. We show that incubation of Arabidopsis (*Arabidopsis thaliana*) cell suspensions with primary alcohols inhibited the induction of two SA-responsive genes, *PATHOGENESIS-RELATED1* and *WRKY38*, in a dose-dependent manner. This inhibitory effect was more pronounced when the primary alcohols were more hydrophobic. Secondary or tertiary alcohols had no inhibitory effect. These results provide compelling arguments for PLD activity being upstream of the induction of these genes by SA. A subsequent study of *n*-butanol effects on the SA-responsive transcriptome identified 1,327 genes differentially expressed upon SA treatment. Strikingly, the SA response of 380 of these genes was inhibited by *n*-butanol but not by *tert*-butanol. A detailed analysis of the regulation of these genes showed that PLD could act both positively and negatively, either on gene induction or gene repression. The overlap with the previously described phosphatidylinositol-4-kinase pathway is discussed.

Plant survival often depends on their ability to acclimate rapidly to various abiotic and biotic environmental stresses. To achieve this, plants utilize several signaling molecules capable of rapidly reprogramming the cellular metabolism. One such molecule is salicylic acid (SA). The most documented function of SA is in mediating plant defense responses to pathogen attack. SA accumulates in infected cells after pathogen recognition, based on the interaction of a pathogen avirulence factor and a cognate plant resistance gene product, resulting in gene-for-gene resistance. In addition to this local accumulation, elevated levels of SA in plant tissues distal from the site of infection induce systemic acquired resistance (SAR) against a broad range of pathogens (Durrant and Dong, 2004). However, SA has been implicated in additional stress responses such as chilling (Scott et al., 2004), heat (Larkindale et al., 2005), osmotic stress (Borsani et al., 2001), or high light (Mateo et al., 2006). SA also plays an important role in programmed cell death (Overmyer et al., 2003), while it also enhances senescence (Buchanan-Wollaston et al., 2005) and induces earlier flowering (Martinez et al., 2004).

Accumulation of SA in local and systemic tissues of infected plants during SAR, as well as exogenous SA treatment, induces a signaling pathway leading to expression of *PATHOGENESIS-RELATED* (*PR*) genes and production of defense proteins. The cytosolic protein NONEXPRESSOR OF PR GENES1 (NPR1) is considered to be the crucial component of this SA pathway. The translocation of NPR1 into the nucleus and its interaction with TGA transcription factors are necessary for *PR1* gene expression in response to

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increased SA levels (Dong, 2004; Durrant and Dong, 2004). Besides TGA factors, several members of the WRKY family of transcription factors have recently been identified as mediators of pathogen-associated transcriptional reprogramming in plants (Eulgem, 2006). WRKY proteins can act as both positive and negative regulators of resistance to pathogen attack (Wang et al., 2006; Xu et al., 2006; Zheng et al., 2007).

While a large effort has been dedicated to elucidating the transcriptional regulation of plant defense genes, little is known about the signaling mechanisms involved in SA responses at the transcriptional level. In tobacco (*Nicotiana tabacum*), SA treatment triggers protein phosphorylation cascades involving MAP kinases (Innes, 2001), specifically the wound-induced protein kinase and SA-induced protein kinase. An increase of cytosolic Ca^{2+} concentration was reported upon SA treatment in BY-2 cells expressing aequorin (Lin et al., 2005).

SA signaling has also been linked with phospholipids and phospholipid-metabolizing enzymes. One of the SA-binding proteins, SABP2, was first identified as a lipase and later its esterase activity hydrolyzing methylsalicylate was described (Forouhar et al., 2005). Silencing the SABP2 gene by RNAi diminished both local resistance and SAR (Kumar et al., 2006). Another example is phosphatidic acid (PA) produced by the activity of phospholipase Dδ (PLDδ), which stimulates NADPH oxidase, leading to both intra- and extracellular oxidative burst. Such oxidative bursts are the first reactions of plants to avirulent pathogens (Xu et al., 2005). Activation of PLC and PLD activities in vitro was described after SA treatment of *Brassica napus* (Profotová et al., 2006). We have recently reported that SA treatment triggers a lipid signaling pathway. In Arabidopsis (*Arabidopsis thaliana*) cell suspensions radiolabeled with $^{33}P_i$, SA addition was accompanied by a rapid increase in labeling of phosphatidylinositol 4-P and phosphatidylinositol 4,5-bisP [PI(4,5)P_2]. These changes in labeling could be inhibited by phenylarsine oxide and wortmannin, both inhibitors of type III phosphatidylinositol 4-kinase (PI4K). A transcriptomic study in response to SA revealed that 14% of SA-regulated genes were inhibited in presence of 30 μM wortmannin (W30; Krinke et al., 2007). This strongly suggested that PI4K is a component of a SA signaling pathway and that the activation of this pathway is necessary for the SA response. Interestingly, a transcription factor of the WRKY family, *WRKY38*, was among the genes whose induction was inhibited by W30 (Krinke et al., 2007).

We were interested to see whether other members of the phospholipid signaling network, namely PLD, are involved in SA signaling. PLD catalyzes the hydrolysis of structural phospholipids to give PA. It is involved in abiotic stress responses mediated by abscisic acid, including cold, drought, and salinity. Specific PLD isoforms have been implicated in the production of, and response to, reactive oxygen species. Furthermore, PLD is associated with the responses to mechanical wounding as well as plant-pathogen interactions. In this latter case the expression of several *PLD* genes and activity of PLD proteins increase after attack of both virulent and avirulent pathogens (Andersson et al., 2006; Anthony et al., 2006; Bargmann et al., 2006; Bargmann and Munnik, 2006).

A unique property of PLDs is their ability to use primary alcohols as an acceptor of a phosphatidyl moiety instead of a water molecule. Thus in the presence of a primary alcohol, PLD catalyzes a transphosphatidylation reaction that leads to the formation of phosphatidylalcohol instead of PA. Secondary and tertiary alcohols are not substrates of the transphosphatidylation reaction (Munnik et al., 1995). Primary alcohols would thus inhibit a cellular response triggered by PLD-produced PA. Using Arabidopsis cell suspensions as a model system, a significant increase in the in vivo PLD activity was detected 45 min after SA addition. Therefore the effects of *n*-butanol on the expression of two SA-induced genes, *PR1* and *WRKY38*, were studied. Finally, based on microarray data, a cross talk between PI4K and PLD pathways in the SA response is discussed.

RESULTS

PLD Activation Can Be Detected in Vivo within the First Hour of SA Treatment

The in vivo PLD activity in cells challenged with SA was measured using the transphosphatidylation reaction. Cells were provided with $^{33}P_i$ to label structural phospholipids such as phosphatidylcholine and phosphatidylethanolamine, the putative substrates of PLD. *n*-Butanol (0.1% [v/v]) was added to the cells 15 min before the addition of SA. Activation of PLD should result in PLD-catalyzed transphosphatidylation, thus yielding phosphatidylbutanol (PtdBut). Lipids were extracted at different times after SA treatment, and separated by thin-layer chromatography (TLC; Supplemental Fig. S1, A and B). At 15 and 30 min after SA treatment a slight increase in the level of PtdBut could be seen in SA-challenged as compared to control cells; the level of PtdBut was significantly higher 45 min after the SA treatment (Fig. 1A). This indicates that PLD activity was triggered between 30 and 45 min after SA treatment. Different concentrations of SA, up to 1 mM, were assayed and it was found that PLD activation after 45 min of SA incubation was dose dependent, with a maximum response observed with 250 μM SA (Fig. 1B). The dose-response curves have also been performed using 0.3% (v/v) or 0.7% (v/v) *n*-butanol as substrate of transphosphatidylation and the same maximum was observed (data not shown).

Induction of *PR1* and *WRKY38* Is Inhibited by Primary Alcohols

Because PLD is activated by SA, we wanted to evaluate the possible involvement of PLD in the SA

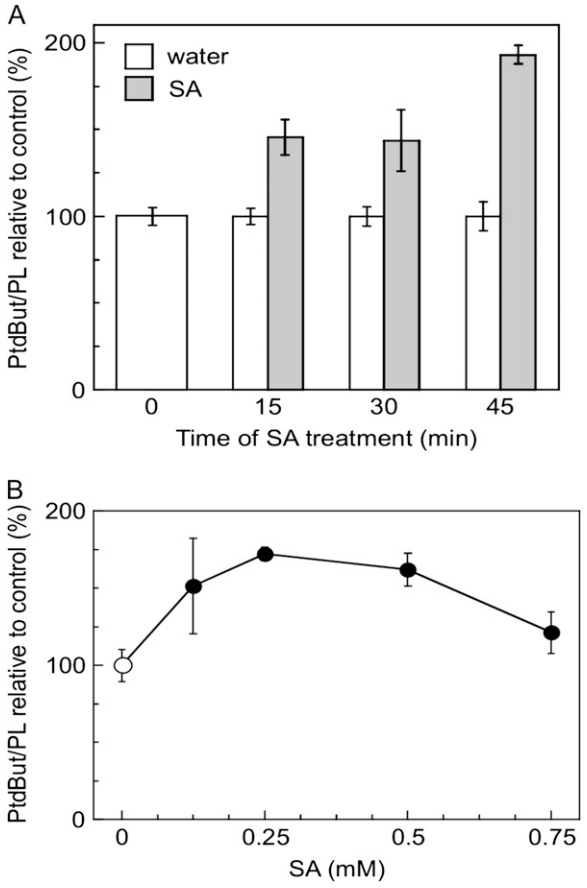

Figure 1. SA activates a PLD activity in vivo. A, Time course of PtdBut production after SA addition. Results of SA-treated samples are compared to water-treated samples at the corresponding time points (100%). B, PtdBut level measured in samples treated with different SA concentrations and compared to the level in water-treated samples. Cells were labeled with $^{33}P_i$ 18 h before addition of SA. *n*-Butanol (0.1% v/v) was added 15 min before SA addition. Lipids were extracted and separated by TLC. Spots corresponding to PtdBut were identified and quantified. The data are means of two independent experiments. PL, Total labeled phospholipids.

signaling pathway. As a marker of the SA response, we used the induction of two genes: *PR1*, the most studied SA-responsive gene, and *WRKY38*, whose SA response we found to be dependent on PI4K (Krinke et al., 2007). The time course of their induction was detected by real-time PCR. As expected, the expression level of *PR1* increased significantly around 5 to 6 h after SA treatment. The induction of *WRKY38* occurred in the first hour and reached a maximum 6 h after SA treatment (Supplemental Fig. S2).

The effects of primary alcohols methanol, ethanol, and *n*-butanol on *PR1* and *WRKY38* induction were therefore monitored 6 h after SA addition (Fig. 2). The alcohols were added at a concentration of 0.1% (v/v). Induction of both genes after 6 h of SA treatment was affected by the addition of primary alcohols. The inhibition increased with the chain length of the primary alcohol, which is expected for a gene regulated through PLD-dependent PA (Munnik et al., 1995). The more hydrophobic the alcohol, the more potent it is as a substrate of the transphosphatidylation reaction. Secondary and tertiary butanol did not affect gene induction (Fig. 2).

Using different concentrations of *n*-butanol or ethanol, we could show by semiquantitative PCR that the inhibition of *PR1* activation was dose dependent (Fig. 3).

Effect of *n*-Butanol versus *tert*-Butanol on the SA-Regulated Transcriptome

n-Butanol is a substrate of PLD while *tert*-butanol is not. We reasoned that if the early PLD activation we were able to detect was part of a signaling pathway leading to the regulation of gene expression, then the genes downstream of PLD-produced PA would have

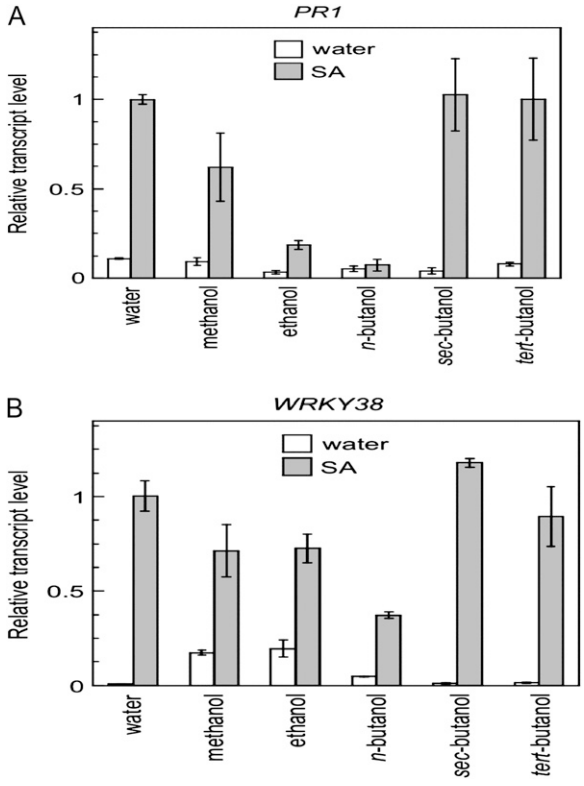

Figure 2. Alcohols differentially affect SA induction of *PR1* and *WRKY38*. Cells were incubated 15 min with different alcohols (final concentration 0.1% [v/v]) prior to SA application and then cultivated for six more hours before RNA isolation. Gene expression was measured by real-time PCR with a relative calibration. Expression levels of *PR1* (A) and *WRKY38* (B) were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The normalized expression level of each gene in cells treated with SA and not treated with any alcohol was arbitrarily set to 1. The data are the means of three real-time PCR reactions from one of two independent experiments with similar results.

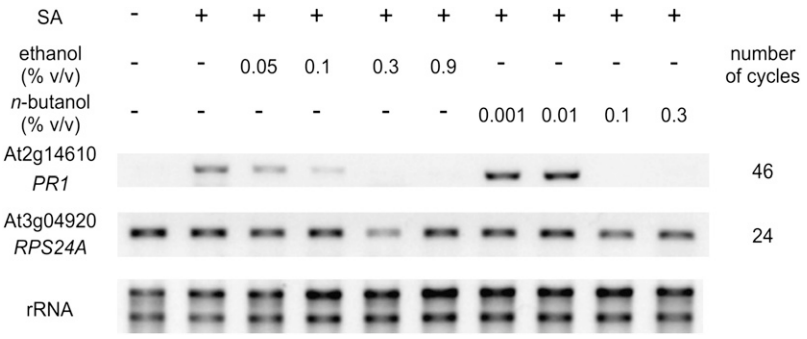

Figure 3. Effect of different concentrations of *n*-butanol or ethanol on *PR1* induction in response to SA. Cells were incubated for 15 min with water or with different concentrations of ethanol or *n*-butanol prior to SA application. After 6 h of SA treatment, RNA was isolated. Gene expression was analyzed by semiquantitative RT-PCR. Cycle number was optimized for each primer pair (indicated on the right). 40S ribosomal protein S24 (At3g04920) was used as a housekeeping gene. The data represent a typical result of two independent experiments. rRNA, Ribosomal RNA.

their SA responses influenced by *n*-butanol while *tert*-butanol would have no effect. To identify genes whose regulation in response to SA is influenced by *n*-butanol but not by *tert*-butanol, a microarray analysis using Complete Arabidopsis Transcriptome MicroArray (CATMA) chips (Lurin et al., 2004) was performed. We chose to extract RNA after a 4 h SA treatment, a time period we already used in a previous transcriptomic study (Krinke et al., 2007), thus enabling a comparison of the two transcriptomes. The RNA samples were prepared from cells that had been treated with either: SA in the presence of 0.1% (v/v) *n*-butanol (hereafter, SANB), SA in the presence of 0.1% (v/v) *tert*-butanol (hereafter, SATB), 0.1% (v/v) *n*-butanol (hereafter, NB), or 0.1% (v/v) *tert*-butanol (hereafter, TB). RNA was extracted from two independent biological repetitions. One dye swap was made per biological repetition (i.e. four hybridizations per comparison) for five combinations: SA versus water, SANB versus SATB, SANB versus SA, SATB versus SA, and NB versus TB. The differentially expressed genes were identified by a paired *t* test on the log2 ratios based on the four hybridizations as described in Krinke et al. (2007). The raw *P* values were adjusted by the Bonferroni methods, which control the family-wise error rate (FWER). NB or SANB is compared to TB or SATB, respectively, to ensure that the effect of NB is not due to a general effect of alcohols. The additional comparison SANB versus SA was done to ensure exclusion of any nonspecific response to alcohols. Only genes differentially expressed and with the transcript level change in the same direction in SANB versus SATB like in SANB versus SA (i.e. induced in both comparisons or repressed in both comparisons) were claimed as being differentially regulated by *n*-butanol versus *tert*-butanol for their SA response.

To identify the genes whose response to SA is affected by NB and not by TB, the following cluster analysis was performed. Among the 1,380 genes that were differentially regulated in response to SA, 1,327 produced a good hybridization in all tested dye swaps. Thirteen genes whose transcript levels were different between SATB and SA were not considered, as they may be regulated by alcohols (Fig. 4). Among the remaining 1,314 genes, 380 could be considered as differentially regulated by *n*-butanol versus *tert*-butanol for their SA response. Of these 380 genes, 238 showed lower transcript levels and 142 showed higher transcript levels in SANB versus SATB. From the 238 genes mentioned, 86 had also lower transcript levels in NB versus TB, while 80 genes from the group of 142 showed higher transcript levels in NB versus TB. For these 166 genes with changes in the same direction in SANB versus SATB and NB versus TB, the effect of SANB versus SATB could be assigned to basal transcript level regulation by PLD but not specifically related to the SA response. For this reason, these 166 genes were excluded from the cluster analysis as they could represent a bias. Among the remaining 214 genes specifically differentially regulated by *n*-butanol versus *tert*-butanol for their SA response, 147 genes were SA induced and 67 were SA repressed. In the group of 147 induced genes, 91 had lower transcript levels in SANB versus SATB, showing an inhibitory effect of *n*-butanol on the SA induction. Among the 67 SA-repressed genes, only six had higher transcript levels in SANB versus SATB, showing an inhibitory effect of *n*-butanol on the SA repression. Possibly underestimated, the number of genes induced via a *n*-butanol-sensitive pathway represents 12% of the SA-induced genes.

To ascertain that the observed numbers are not a product of random events, a test of colocalization of the two phenomena of regulation was performed, i.e. colocalization of the SA-regulated gene expression (SA versus water comparison) with the regulation of SA response by NB (SANB versus SATB comparison). The genes regulated nonspecifically by alcohols in their response to SA (i.e. genes with a transcript level change in the SATB versus SA comparison or with a different behavior in the SANB versus SATB and SANB versus SA comparisons) were removed from the analysis as well as the genes showing the same mode of regulation even in the nonstimulated (water-treated) cells (i.e. in the NB versus TB comparison) as these may artificially increase the observed overlap of regulation modes (since they represent false positives). Each gene among the remaining ones could be unambiguously classified into one of the four groups: genes with a transcript level change both in the SA versus water and SANB versus SATB comparisons, genes with a transcript level change only in one comparison,

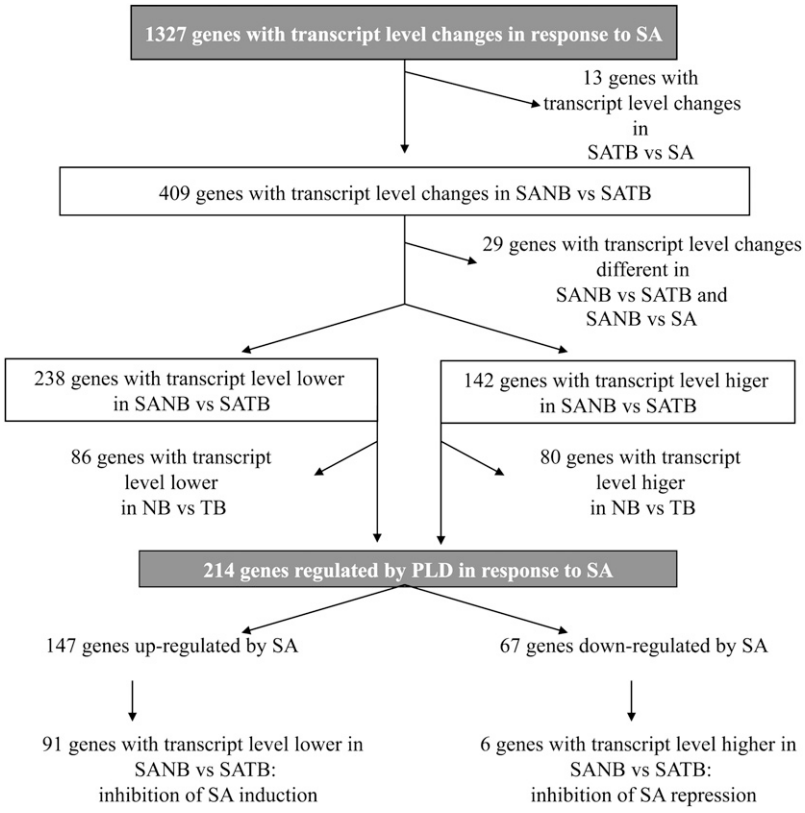

Figure 4. Determination of SA-regulated genes downstream of a *n*-butanol-sensitive pathway. Among the 4,022 genes that showed differential gene expression in one of the five tested comparisons, 214 showed transcript level changes both in the SA versus water comparison and in the same direction in the SANB versus SATB comparison as in the SANB versus SA comparison. For these genes the transcript levels did not change in the same direction in the NB versus TB comparison as in the SANB versus SATB comparison. Hence these genes are probably SA regulated via the PLD pathway. Of these genes, 147 were up-regulated and 67 were down-regulated by SA treatment. Ninety-seven genes out of the 214 showed a positive action of PLD on their SA response.

and genes with no transcript level change in any of the two comparisons. Probabilities that the observed distributions were due to random events (calculated by the Fisher's test) are given in Table I. Three of the four possible colocalizations were not a product of coincidence ($\alpha = 0.05$), showing that the regulation of gene expression by SA and regulation of the same response by *n*-butanol colocalize in the transcriptome much more than would be expected in case of a random distribution. Only the group of SA-repressed genes positively regulated by a *n*-butanol-sensitive pathway may result from random overlap of the two modes of regulation of gene expression.

The first 10 genes from the category of SA-induced genes positively regulated by a *n*-butanol-sensitive pathway and from categories of SA-regulated genes negatively regulated by a *n*-butanol-sensitive pathway ranked by decreasing degree of their repression or induction by NB are listed in Table II. The complete list of 97 genes positively and 117 genes negatively regulated by a *n*-butanol-sensitive pathway in response to SA is provided as Supplemental Table S1.

Table I. *Colocalization analysis of regulation by SA with the regulation by a n-butanol-sensitive pathway in response to SA on the whole transcriptome scale*

Genes regulated in the SATB versus SA comparison or with different behavior in the SANB versus SATB and SANB versus SA comparisons and genes regulated in the same way in the SANB versus SATB as in the NB versus TB comparison were removed from the analysis. The remaining 17,343 genes were classified with respect to their regulation in the SA versus water and SANB versus SATB comparisons. The expected number of genes for each cluster was calculated and compared to the actual number observed in the analysis. *P* values are those of a Fisher's test. Categories printed in bold are overrepresented in the experimental data. d_{obs}, Observed distribution; d_{theor}, theoretical distribution.

SA Regulation	SANB versus SATB	Impact of *n*-Butanol-Sensitive Pathway on the SA Response	No. of Genes		P (d_{obs} = d_{theor})
			Observed	Theoretical	
Induced	<	Positive	**91**	**11**	**7.4e–58**
	>	Negative	**56**	**9**	**8.6e–29**
Repressed	>	Positive	6	7	1.0
	<	Negative	**61**	**8**	**1.0e–34**

Table II. *List of SA-regulated genes most influenced by NB treatment, ranked by decreasing degree of repression or induction by NB*

The difference in transcript level was significant in each case ($\alpha = 0.05$). AGI, Arabidopsis Genome Initiative gene index; *I*, average signal intensity.

Protein	Function	AGI	$\log_2 I_{SANB}/I_{SATB}$	$\log_2 I_{SA}/I_{water}$
Induced genes, positively regulated by a *n*-butanol-sensitive pathway[a]				
Expressed protein	Unknown	At1g31580	−3.85	3.66
Glycerophosphoryl diester phosphodiesterase family protein	Kinase activity	At1g66970	−3.54	2.91
WRKY66	Transcription factor activity	At1g80590	−2.91	4.02
WRKY38	Transcription factor activity	At5g22570	−2.77	4.59
Heavy-metal-associated domain-containing protein	Metal ion binding	At5g26690	−2.56	2.88
AAA-type ATPase family protein	ATPase activity	At3g28540	−2.44	2.62
D-3-Phosphoglycerate dehydrogenase	Phosphoglycerate dehydrogenase activity	At1g17745	−2.26	2.96
Cytochrome P450 monooxygenase 83B1	Cytochrome P450 activity	At4g31500	−2.09	1.63
Expressed protein	Unknown	At1g19960	−2.04	2.81
Protein phosphatase 2C, putative	Protein phosphatase type 2C activity	At1g34750	−2.03	2.15
Induced genes, negatively regulated by a *n*-butanol-sensitive pathway[b]				
60S ribosomal protein L10 (RPL10C)	Structural constituent of ribosome	At1g66580	2.07	0.92
UDP-glucoronosyl/UDP-glucosyl transferase family protein	Transferase activity, transferring glycosyl groups	At2g15490	1.94	2.42
Dehydrin xero2/low-temperature-induced protein LTI30	Unknown	At3g50970	1.84	1.87
Hydrophobic protein, putative/low temperature and salt-responsive protein, putative	Unknown	At4g30660	1.69	0.81
Transcription initiation factor IIA γ chain/TFIIA-γ	RNA polymerase II transcription factor activity	At4g24440	1.61	0.67
Complex 1 family protein/LVR family protein	Catalytic activity	At2g39725	1.61	0.73
Pseudogene, Gly-rich protein	Structural molecule activity	At2g36120	1.53	0.84
Expressed protein	ATP binding	At1g15230	1.50	1.06
Drought-responsive family protein	Unknown	At3g06760	1.45	0.84
Transcription factor, putative	Transcription factor activity	At1g56170	1.34	1.15
Repressed genes, negatively regulated by a *n*-butanol-sensitive pathway[c]				
Expressed protein	Unknown	At5g42090	−1.96	−1.35
SEC14 cytosolic factor family protein/ phosphoglyceride transfer family protein	Transporter activity	At1g72150	−1.86	−1.16
Pyrophosphate-Fru-6-P 1-phosphotransferase β-subunit, putative/pyrophosphate-dependent 6-phosphofructose-1-kinase, putative	6-Phosphofructokinase activity	At4g04040	−1.83	−1.35
Cellulose synthase, catalytic subunit (Ath-B)	Cellulose synthase activity	At5g05170	−1.59	−2.04
RNA polymerase α chain	Unknown	AtCg00740	−1.55	−1.90
Amino acid transporter family protein	Amino acid permease activity	At3g30390	−1.46	−0.88
Ribosomal protein L2	Unknown	AtCg00830	−1.37	−1.14
Expressed protein	Unknown	At3g49990	−1.36	−0.82
Endomembrane protein 70, putative	Transporter activity	At4g12650	−1.31	−1.57
ATP synthase CF1 β chain	Unknown	AtCg00480	−1.29	−0.78

[a]Top 10 genes positively regulated by a *n*-butanol-sensitive pathway in their SA induction. [b]Top 10 genes negatively regulated by a *n*-butanol-sensitive pathway in their SA induction. [c]Top 10 genes negatively regulated by a *n*-butanol-sensitive pathway in their SA repression.

To confirm the data obtained by DNA microarray analysis we selected genes whose response to SA was inhibited or enhanced by *n*-butanol. Using real-time PCR we verified that nine genes, including *NPR1*, were strongly induced by SA while this induction was largely diminished or totally suppressed by *n*-butanol (Supplemental Fig. S3A) and that three genes were induced by SA while this induction was enhanced by *n*-butanol (Supplemental Fig. S3B).

NPR1-Dependent Genes Are Overrepresented among SA-Regulated Genes Inhibited by *n*-Butanol

NPR1, an important transcription regulator of the SA response, was among the genes whose regulation by the *n*-butanol-sensitive pathway was confirmed by real-time PCR (Supplemental Fig. S3A). Therefore it was interesting to investigate whether there is a significant overlap between NPR1-regulated genes and

genes regulated by the *n*-butanol-sensitive pathway. One publication reported a list of NPR1-dependent genes in response to SA (Wang et al., 2005). The authors treated 4-week-old Arabidopsis plants with 500 μM SA for 24 h and then identified the subset of genes positively regulated by NPR1 using Affymetrix GeneChip containing probes for 8,200 genes. To be able to compare the two different experiments, a set of genes common to both platforms was generated (245 SA induced and 214 SA repressed genes in our CATMA experiment had a probe on the Affymetrix AG chip; Supplemental Table S2). Genes (separately SA induced and SA repressed) were divided into four categories with respect to their regulation by NPR1 and *n*-butanol-sensitive pathway (positive and negative action of *n*-butanol-sensitive pathway was considered separately). Probabilities that the observed distributions were due to random events were calculated by the Fisher's test. Only the overlaps between genes positively regulated by the *n*-butanol-sensitive pathway and NPR1-dependent genes (either SA induced or SA repressed) were significantly overrepresented (Table III). The result of this metaanalysis is in perfect agreement with the real-time PCR confirmation of positive regulation of *NPR1* by the *n*-butanol-sensitive pathway.

Comparison of genes regulated via a *n*-butanol-sensitive pathway with the set of genes positively regulated by NPR1 (Wang et al., 2005) did not reveal overrepresentation of a specific type of proteins in the identified overlaps (Supplemental Table S2). It has to be noted that *PR1* could not be identified as a SA-regulated gene in our CATMA experiment because there is no probe for it on the CATMA gene chip. Therefore this interesting gene cannot be listed in the Supplemental Table S2.

Common cis-Elements in the Promoters of Genes Regulated by *n*-Butanol versus *tert*-Butanol in Response to SA

As we identified a group of genes regulated by a *n*-butanol-sensitive pathway in their SA response, it was interesting to determine whether any of the promoter motifs specific for the SA-regulated genes (Krinke et al., 2007) are overrepresented in this group. Genes with unambiguous probe-to-gene assignment were extracted from Supplemental Table S1. For each promoter motif identified by Krinke et al. (2007) as overrepresented in SA-responsive genes, its frequency in the group of genes regulated by a *n*-butanol-sensitive pathway was compared to its frequency in the subset of SA-regulated genes (either induced or repressed depending on the tested regulon) using a general bootstrapping method with 200 random trials. No motifs were differently represented in the cluster of SA-repressed genes either positively or negatively regulated by the *n*-butanol-sensitive pathway. Significantly over- and underrepresented motifs in promoters of SA-induced genes are listed in Table IV. One motif, the W-box (TTGACTT/TTGACY) motif, was significantly overrepresented in the group of genes induced by SA via the positively acting *n*-butanol-sensitive pathway. These data suggest that WRKY transcription factors may be situated downstream of the positive action of the *n*-butanol-sensitive pathway. Two other motifs, similar to LTRE and CBF1 binding sites, were overrepresented in the group of SA-induced genes negatively regulated by the *n*-butanol-sensitive pathway.

Overlap of W30-Sensitive and *n*-Butanol-Sensitive SA Transcriptomes

In a previous study, we had shown that PI4K was activated in response to SA. We had performed a transcriptomic study of the SA response in the presence of W30. W30 inhibits PI4K, but no inhibition occurs at 1 μM (W1); W1 was used as a negative control (Krinke et al., 2007). We wanted to know whether the SA-responsive transcriptome regulated through a *n*-butanol-sensitive pathway overlapped with the SA-responsive transcriptome regulated through a mechanism sensitive to W30, most likely on the level of PI4K activity (Krinke et al., 2007). To be able to make the

Table III. *Colocalization metaanalysis of gene regulation by NPR1 with the regulation by a n-butanol-sensitive pathway in response to SA on the whole transcriptome scale*

Only genes present on both microarray platforms (CATMA and Affymetrix AG) and differentially expressed after SA treatment in the CATMA experiment were considered (245 SA-induced and 214 SA-repressed genes). The genes were classified with respect to their regulation by *n*-butanol-sensitive pathway and by NPR1 in response to SA. The expected number of genes for each cluster was calculated and compared to the actual number observed in the analysis. *P* values are those of a Fisher's test. Categories printed in bold are overrepresented in the experimental data (only overlaps, i.e. genes regulated by both pathways, are listed for clarity). d_{obs}, Observed distribution; d_{theor}, theoretical distribution.

SA Regulation	Impact of *n*-Butanol-Sensitive Pathway on the SA Response	No. of Genes in the Overlap		P (d_{obs} = d_{theor})
		Observed	Theoretical	
Induced	Positive	**8**	**3**	**2.7e–3**
	Negative	1	3	4.8e–1
Repressed	Positive	**2**	**0**	**6.0e–3**
	Negative	0	0	1.0

Table IV. *cis-Elements identified in the promoters of the subgroup of genes induced by SA via the n-butanol-sensitive pathway*

The analysis was performed on a subset of genes with unambiguous probe-to-gene assignments. The number of appearances of each motif in promoters (−1,000 bp) of the cluster of 84 genes induced by SA via the positive action of a *n*-butanol-sensitive pathway ($f_{obs,p}$) and in promoters of the cluster of 55 genes induced by SA via the negative action of a *n*-butanol-sensitive pathway ($f_{obs,n}$) was compared to its frequency in promoters of 681 SA-induced genes (f_{theor}) by a general bootstrapping technique. n.s.d., The observed frequency is not significantly different from the theoretical frequency ($P \geq 0.05$). Motifs were annotated according to the Riken Arabidopsis Genome Encyclopedia (Sakurai et al., 2005). R, A or G; Y, C or T; W, A or T.

Motif Description	Searched Motif	f_{theor}	Positive Action of a *n*-Butanol-Sensitive Pathway		Negative Action of a *n*-Butanol-Sensitive Pathway	
			$f_{obs,p}$	$P(f_{obs,p} = f_{theor})$	$f_{obs,n}$	$P(f_{obs,n} = f_{theor})$
I-box, MYB-like family	GATAAG	0.64	n.s.d.	–	0.42	2.2e–2
ASF-1-like, TGA family	TGACGTG	0.12	0.05	2.0e–2	n.s.d.	–
W-box, WRKY family	TTGACY	1.51	2.02	8.4e–5	n.s.d.	–
W-box-like, WRKY family	TTGACTT	0.43	0.77	2.9e–7	n.s.d.	–
bZip	ACGT	5.13	4.19	9.1e–3	n.s.d.	–
G-box bZip variants	YACGTG	0.77	0.33	8.8e–4	n.s.d.	–
Similar to LTRE	RGGCCY	0.88	0.60	4.0e–2	1.31	1.6e–2
CBF1 binding site	CCGWC	1.17	n.s.d.	–	1.39	3.2e–2
SORLIP2, light responsive	TGGGCC	0.49	0.33	4.2e–2	n.s.d.	–

connection between the PI4K activity and the *n*-butanol-sensitive pathway (most likely corresponding to PLD activity as discussed below) in the regulation of SA transcriptome, we checked whether the groups of genes either regulated by W30 or by *n*-butanol shared a common overlap and whether the overlap was more than a simple coincidence. A common base for the overlap analysis was constructed according to the following rules: The genes had to be regulated in the same way in both microarray experiments (i.e. induced by SA in both or repressed by SA in both). Moreover, genes removed from either analysis for their nonspecific regulation were omitted from the overlap analysis. This concerns genes regulated by methanol or W1 in their SA response, genes regulated by W30 in the same way in water-treated as in SA-treated conditions (Krinke et al., 2007), genes regulated by TB in their SA response, and genes regulated by NB in the same way in water-treated as in SA-treated conditions (this study). This led to the construction of a set of 182 SA-induced genes and a set of 75 SA-repressed genes whose mode of regulation in response to SA (i.e. positive or negative regulation of SA induction or SA repression by either pathway) was unambiguous. For the SA-repressed genes, the numbers of genes in the considered overlapping clusters were too low to perform a statistical analysis. For SA-induced genes, four colocalization analyses could be designed (Table V).

Based on a Fisher's test ($\alpha = 0.05$), the overlaps of both positive regulations and both negative regulations were overrepresented (when compared to random/theoretical distribution) while overlaps of one negative mode of regulation with one positive mode of regulation were underrepresented. Expression profiles of genes from overlaps of clusters regulated via a W30-sensitive pathway and via a *n*-butanol-sensitive pathway in response to SA are given in Supplemental Table S3. We verified by semiquantitative reverse transcription (RT)-PCR that the induction by SA of some W30-inhibited genes were also inhibited by *n*-butanol (Fig. 5A). *PR1* is not listed in Supplemental Table S3 because it is not represented in the CATMA array. However, because of its importance as a marker of the SA response, *PR1* gene expression was also analyzed by semiquantitative RT-PCR; its SA induction was inhibited both by W30 and by *n*-butanol (Fig. 5B). *NPR1* expression was then assayed, by real-time PCR. While its induction by SA was inhibited by *n*-butanol, it was not inhibited by W30 (Fig. 5C).

DISCUSSION

PLD Activation in Response to SA

Substantial progress has been made in our understanding of the signaling events activated by plant responses to SA (Durrant and Dong, 2004; Bostock, 2005). However, much of the effort was dedicated to the identification of transcriptional regulators (Eulgem, 2006; Thibaud-Nissen et al., 2006), while early signaling events of the SA pathway remain to be unraveled. In our previous study, we reported dramatic changes in phosphoinositide levels dependent on the PI4K activity as an immediate response to SA treatment (Krinke et al., 2007). This research has identified PLD as another component of the SA signaling pathway. Using *n*-butanol as a PLD substrate we could show that a PLD was activated a few minutes after treatment, with a peak around 45 min after SA addi-

Table V. *Analyses of overlaps of different modes of regulation within the SA-induced transcriptome*

The 182 induced genes selected were classified with respect to their regulation in the SAW30 versus SAW1 (a W30-sensitive pathway) and SANB versus SATB (a *n*-butanol-sensitive pathway) comparisons. The expected number of genes for each overlap was calculated and compared to the observed number. *P* values are those of a Fisher's test. Categories printed in bold are overrepresented while those printed in italics are underrepresented in the experimental data. d_{obs}, Observed distribution; d_{theor}, theoretical distribution.

SA Regulation	SAW30 versus SAW1	SANB versus SATB	No. of Genes		$P(d_{obs} = d_{theor})$
			Observed	Theoretical	
SA induced	<	<	**33**	**16**	**8.8e–9**
		>	*0*	*4*	*2.1e–2*
	>	<	0	2	1.8e–1
		>	**2**	**0**	**4.2e–2**

tion. This time course of PLD activation is similar to that observed for the response to abscisic acid (Hallouin et al., 2002) in Arabidopsis cells. The PLD activation was dependent on the dose of SA, with a maximum of response obtained with 250 μM SA. Arabidopsis genome encodes 12 different *PLD* isoforms (Wang, 2004). None of them was significantly induced by SA treatment, even after 1 h (data not shown). The PLD isoform involved in the SA response is likely to be activated at the protein level, either by increased translation or by activation of the protein already present.

A *n*-Butanol-Sensitive Pathway Is Upstream of the SA-Triggered NPR1 Pathway

If some genes were responsive to SA via PLD-produced PA, then the addition of *n*-butanol would

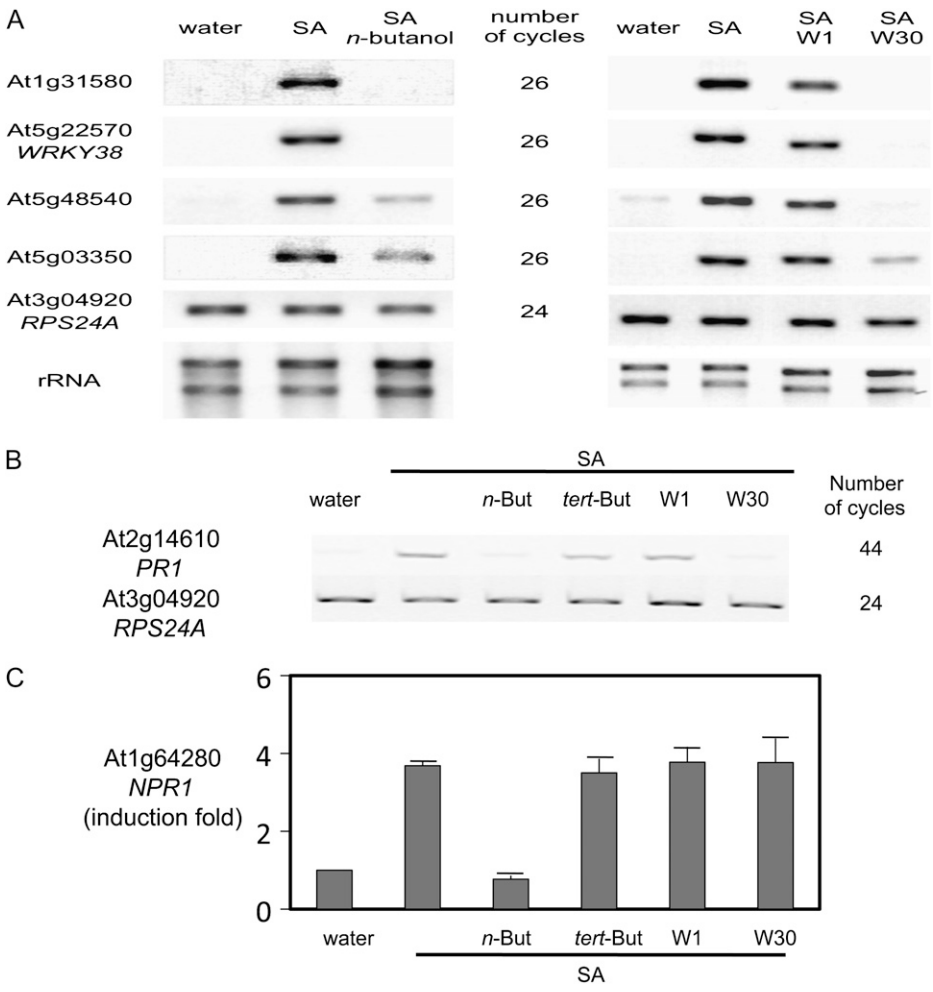

Figure 5. Regulation by W30 and *n*-butanol of SA-induced genes. A, Expression of At1g31580, *WRKY38*, At5g48540, and At5g03350 monitored by semiquantitative RT-PCR. In the left section, RNAs were isolated 6 h after SA addition; in the right section, RNAs were isolated 4 h after SA addition. rRNA, Ribosomal RNA. B, Expression of *PR1* monitored by semiquantitative RT-PCR. RNAs were isolated 4 h after SA addition. C, Expression of *NPR1* monitored by real-time PCR. RNAs were isolated 4 h after SA addition. In A and B, the PCR cycle number was optimized for each primer pair. 40S ribosomal protein S24 (At3g04920) was used as a housekeeping gene. The data represent a typical result of two independent experiments. In A, B, and C, cells were pretreated with either with 0.1% (v/v) *n*-butanol or W1 or W30 for 15 min before SA addition. *n*-But, *n*-Butanol; *tert*-But, *tert*-butanol.

inhibit the gene response while *tert*-butanol would not. We were able to identify 97 genes whose response to SA was inhibited by *n*-butanol and was not affected by *tert*-butanol; these genes were positively regulated by a *n*-butanol-sensitive pathway in response to SA. Besides we identified 117 genes whose response to SA was enhanced by *n*-butanol and was not affected by *tert*-butanol; these genes were negatively regulated by a *n*-butanol-sensitive pathway in response to SA. The cluster of genes positively regulated by the *n*-butanol-sensitive pathway contains transcription factors and *PR* genes important for SAR development (e.g. *NPR1*, *NIMIN1*, *NIMIN2*, *WRKY38*, *WRKY66*, or *TGA1*). Crossing our results with the microarray data identifying NPR1-dependent genes (Wang et al., 2005), we could show that these genes are overrepresented in the cluster of SA-regulated genes positively regulated by the *n*-butanol-sensitive pathway. Clusters of genes negatively regulated by the *n*-butanol-sensitive pathway, both SA induced and SA repressed, comprised many genes involved in transcription (e.g. transcription factors including SA-induced *WRKY51*, transcription initiation factor, phosphoribulokinase/uridine kinase-related protein, and RNA polymerase), but even more of those involved in translation (e.g. amino acid transporter family protein, a tRNA synthetase, various ribosomal proteins, translation initiation factor, elongation factor EF-1α). Blocking these resource-demanding metabolic processes through the negative action of *n*-butanol-sensitive pathway may ensure a tighter control of the developmental processes related to the SA response.

Taken together our results show that a *n*-butanol-sensitive pathway is important for the SA response and that the NPR1 pathway, which has a key role in SAR, is activated downstream of the *n*-butanol-sensitive pathway. Interestingly, the regulation of *NPR1* transcription depends on WRKY transcription factors (Yu et al., 2001). Besides, there is an overrepresentation of W-boxes, cis-elements recognized by WRKY transcription factors, in the promoters of SA-induced genes that are positively regulated via the *n*-butanol-sensitive pathway. Therefore the regulation of *NPR1* transcription may be ensured by the *n*-butanol-sensitive pathway through WRKY transcription factors. This would correspond to the following sequence of events: SA → *n*-butanol-sensitive pathway → WRKY → NPR1. In addition, the *n*-butanol-sensitive pathway could act on the activation NPR1 protein. NPR1 transcription factor must interact with TGA proteins in their reduced forms to be active (Dong, 2004). In SAR, SA is known to trigger an initial oxidative burst. However, SA is also thought to be required for the establishment of the subsequent reducing conditions, perhaps via the activation of genes encoding antioxidants (Dong, 2004). It would be interesting to investigate whether those redox changes are upstream or downstream of the *n*-butanol-sensitive pathway. It can be noted that transcription of several genes involved in cell redox status, such as putative glutathione *S*-transferase, glutaredoxin, L-ascorbate peroxidase, and several thioredoxins, is influenced by *n*-butanol in response to SA, suggesting a role for the *n*-butanol-sensitive pathway in the control of the cell redox status. Finally, we could note that even though the overrepresentation of genes in the overlaps between the *n*-butanol-sensitive pathway and NPR1 pathway is statistically significant, it represents only a minor portion of *n*-butanol-sensitive pathway regulated genes. This leaves space for alternative *n*-butanol-sensitive pathways parallel to that of NPR1.

The SA-Triggered *n*-Butanol-Sensitive Pathway Is Likely to Be a PLD Pathway

Because *n*-butanol is a substrate of PLD while *tert*-butanol is not, this raises a question about whether the *n*-butanol effect is specific. The question is whether each difference between the effects of *n*-butanol versus *tert*-butanol can be interpreted as a consequence of the different production of PA by PLD. Other effects of alcohols have been described. For example small alcohols can make membranes more fluid (Dickey and Faller, 2007); however, no differences are expected between primary, secondary, or tertiary alcohols on membrane fluidity. Alcohols also have some effects on G proteins: Primary and secondary alcohols, but not tertiary alcohols, could activate G proteins. In the real-time PCR experiments with *PR1* and *WRKY38*, the inhibition observed with *n*-butanol was not seen with secondary alcohols, implying that the effect seen with primary alcohols was due to inhibition of PLD-produced PA and not due to G-protein activation. In addition, if the effect of *n*-butanol on the gene expression was due to G-protein activation, the effect would also be detected in cells not challenged with SA. However, when we selected the genes as being SA driven via a *n*-butanol-sensitive pathway, we took out the genes for which the effect of *n*-butanol was the same with or without SA. Finally, it has been reported that *n*-butanol, but not *tert*-butanol, can depolymerize microtubules in tobacco cells even in vitro (Hirase et al., 2006). Such an effect could alter gene expression and interfere with the observed inhibition of the SA response. Here again, if the effect of *n*-butanol on gene expression was due to microtubule depolymerization, the effect would also be detected in cells not challenged with SA. This is not the case for the genes we identified as SA driven via a *n*-butanol-sensitive pathway. To further test this, we treated cells with 5 μM oryzalin, a microtubule-depolymerizing drug that is effective in plants (Binet et al., 2001). After 6 h of SA treatment, the expression of *PR1* was only slightly lowered and that of *WRKY38* was not affected at all, thus excluding the possibility that *n*-butanol suppresses gene expression through PLD-independent microtubule depolymerization (data not shown). Finally, the fact that the inhibitory effect of aliphatic primary alcohols on the SA induction of *WRKY38* and *PR1* genes increased with the alkyl chain length is charac-

teristic of PLD action. The SA-triggered *n*-butanol-sensitive pathway is therefore likely to be a PLD pathway.

Are PI4K and PLD Pathways Overlapping in the SA Response?

An important overlap was observed when comparing the W30-sensitive pathway-regulated and PLD-regulated clusters. Genes positively regulated by both pathways were significantly overrepresented in the cluster of SA-induced genes. On the other hand, mixed overlaps between one positive and one negative mode of regulation were underrepresented. Among the SA-induced genes positively regulated by both pathways was *PR1*. This would plead for PI4K and PLD being in the same signaling pathway, leading to the activation of the same target genes. PI4K is activated in the first minutes of SA treatment while PLD is activated later, after 45 min. It is concomitant with the peak of PI(4,5)P_2. Most Arabidopsis PLDs, but not all, are PI(4,5)P_2 dependent (Wang, 2004). Therefore some genes can be activated by a PI(4,5)P_2-dependent PLD and others, like *NPR1*, by a PI(4,5)P_2-independent PLD. This suggests that different PLD isoforms are activated upon SA elicitation; some would be downstream of the PI(4,5)P_2 increase while others would not. The fact that multiple PLD isoforms might be activated upon a stress has already been described. In Arabidopsis, both PLDα1 and PLDδ were found to be activated in response to salt stress (Bargmann et al., 2009). Plants or suspension cells mutated in PI4K isoforms would be the tool of choice to further investigate the activation of particular PLD isoforms downstream of the PI4K activation within the SA response.

MATERIALS AND METHODS

Cell Cultures

Cell suspensions of Arabidopsis (*Arabidopsis thaliana*), ecotype Columbia-0, and their maintenance are described by Vergnolle et al. (2005). Experiments were performed on 5-d-old cultures that correspond to the end of the exponential phase (cultures were multiplied every seventh day).

SA Treatment and Lipid Analysis

Cells (7 mL of cell suspension; 1 g fresh weight) were treated with 250 μM SA unless stated otherwise. SA (sodium salt) was purchased from Sigma-Aldrich and did not show any buffering or pH-modifying capacity up to 2 mM. Cells were labeled by $^{33}P_i$ according to the procedure previously described by Ruelland et al. (2002). Total lipids were extracted and separated by TLC. PA or PtdBut were separated from structural phospholipids using the solvent system composed of an upper phase of ethyl acetate:isooctane:acetic acid: water (12:2:3:10 [v/v]; de Vrije and Munnik, 1997). Radiolabeled spots were quantified by autoradiography using a Storm phosphorimager (Amersham Biosciences). Separated phospholipids were identified by comigration with authentic nonlabeled standards visualized by primuline staining (under UV light) or by phosphate staining.

RNA Extraction and Quantitative RT-PCR Analysis

Cells (0.6 mL of cell suspension; 85 mg fresh weight) were filtered and immediately frozen in liquid nitrogen. Cells were ground in liquid nitrogen and the resulting powder was transferred to 500 μL of TRI Reagent from Sigma-Aldrich. RNA was extracted using the Trizol extraction method according to the manufacturer's protocol. Dried RNA pellets were dissolved in 100 μL of sterile water. RNA concentration was determined by the A_{260} measured using a NanoDrop ND 1000 (NanoDrop Technologies) spectrophotometer. RNA quality was checked by horizontal electrophoresis using 1% (w/v) agarose gels, 0.5× TBE buffer (65 mM Tris; 22.5 mM boric acid; 1.25 mM EDTA), and 0.5 μg mL^{-1} ethidium bromide.

One microgram of total RNA was treated with DNase I (Sigma-Aldrich) and reverse transcribed using the iScript cDNA synthesis kit according to the manufacturer's instructions. An equivalent of 25 ng of total RNA was amplified with 0.3 μM gene-specific primers designed by Vector NTI software (version 10.3.0). The gene encoding a 40S ribosomal protein S24 (At3g04920) was used as a housekeeping gene. Ten- and 100-fold dilutions of one reference sample were prepared to determine the efficiency of real-time PCR. Amplification was carried out using a Bio-Rad MJ Mini Opticon thermal cycler and iQ SYBR Green Supermix according to the manufacturer's instructions. Annealing temperature was 57°C for all primer pairs. Threshold cycles (c_T) for each sample were determined with Opticon Monitor software (version 3.1). Real-time PCR efficiency was determined from the slope of the plot of c_T against log of dilution of the reference sample. Gene expression in each sample was normalized to the expression of the housekeeping gene.

Semiquantitative RT-PCR Analysis

Cells (7 mL of cell suspension; 1 g fresh weight) were filtered and immediately frozen in liquid nitrogen. RNA was extracted using the phenol/chloroform extraction described in Vergnolle et al. (2005). For the semiquantitative RT-PCR, 1 μg of total RNA was treated with DNase I (Sigma-Aldrich) and reverse transcribed using the Omniscript reverse transcriptase kit from Qiagen and oligo(dT)$_{15}$ primers according to the supplier's instructions. An equivalent of 40 ng of total RNA was amplified with 0.6 μM gene-specific primer pairs. The gene encoding a 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. Annealing temperature was 53°C for all primer pairs. A suitable number of PCR cycles were used for each primer pair. PCR was separated on horizontal electrophoresis in 1% (w/v) agarose gels, 0.5× TBE buffer, and 0.5 μg mL^{-1} ethidium bromide.

Transcriptome Studies

The microarray analysis was carried out at the Unité de Recherche en Génomique Végétale using the CATMA array (Crowe et al., 2003; Hilson et al., 2004), containing 24,576 gene-specific tags from Arabidopsis. RNA samples from two independent biological replicates were used. For each biological repetition, RNA samples for a condition were obtained by pooling RNA from three independent extractions (1 mg fresh weight per extraction). For each comparison, one technical replication with fluorochrome reversal was performed for each biological replicate (i.e. four hybridizations per comparison). The RT of RNA in the presence of Cy3-dUTP or Cy5-dUTP (PerkinElmer-NEN Life Science Products), the hybridization of labeled samples to the slides, and the scanning of the slides were performed as described in Lurin et al. (2004).

Statistical Analysis of Microarray Data

Experiments were designed with the statistics group of the Unité de Recherche en Génomique Végétale. The statistical analysis was based on two dye swaps (i.e. four arrays, each containing 24,576 gene-specific tags and 384 controls). For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). No background was subtracted. In the following description, log ratio refers to the differential expression between two conditions. It is either $\log_2$(red/green) or $\log_2$(green/red) according to the experimental design. Array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered badly formed features. Then, we performed a global intensity-dependent normalization using the LOESS procedure to correct the dye bias. Finally, for each block, the log ratio median calculated over the values for the entire block was subtracted from each individual log ratio value to correct print tip effects on each metablock. To determine differentially expressed genes, we performed a paired *t* test on the log ratios, assuming that the variance of the log ratios was the same for all genes. Spots displaying extreme variance (too small or too large) were excluded. The raw *P*

values were adjusted by the Bonferroni method, which controls the FWER. Genes with an FWER < 5% were considered as differentially expressed.

Data Deposition

Microarray data from this article were deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE9695) and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: AU07-01_PLD-SA) according to MIAME standards.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. TLC plates showing that SA activates PLD in vivo.

Supplemental Figure S2. Time course of SA induction of *PR1* and *WRKY38*.

Supplemental Figure S3. Confirmation of microarray results on an independent set of nonpooled RNAs.

Supplemental Table S1. Expression profiles of genes regulated either positively or negatively by a *n*-butanol-sensitive pathway in response to SA.

Supplemental Table S2. Overlaps between clusters regulated positively by NPR1 and either positively or negatively by a *n*-butanol-sensitive pathway in response to SA.

Supplemental Table S3. Expression profiles of genes from overlaps of clusters regulated via a W30-sensitive pathway and via *n*-butanol-sensitive pathway within the SA-induced transcriptome.

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LITERATURE CITED

Andersson MX, Kourtchenko O, Dangl JL, Mackey D, Ellerstrom M (2006) Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*. Plant J **47:** 947–959

Anthony RG, Khan S, Costa J, Pais MS, Bogre L (2006) The Arabidopsis protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. J Biol Chem **281:** 37536–37546

Bargmann BO, Laxalt AM, Riet BT, Schouten E, van Leeuwen W, Dekker HL, de Koster CG, Haring MA, Munnik T (2006) LePLDβ1 activation and relocalization in suspension-cultured tomato cells treated with xylanase. Plant J **45:** 358–368

Bargmann BO, Laxalt AM, ter Riet B, van Schooten B, Merquiol E, Testerink C, Haring MA, Bartels D, Munnik T (2009) Multiple PLDs required for high salinity and water deficit tolerance in plants. Plant Cell Physiol **50:** 78–89

Bargmann BO, Munnik T (2006) The role of phospholipase D in plant stress responses. Curr Opin Plant Biol **9:** 515–522

Binet MN, Humbert C, Lecourieux D, Vantard M, Pugin A (2001) Disruption of microtubular cytoskeleton induced by cryptogein, an elicitor of hypersensitive response in tobacco cells. Plant Physiol **125:** 564–572

Borsani O, Valpuesta V, Botella MA (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. Plant Physiol **126:** 1024–1030

Bostock RM (2005) Signal crosstalk and induced resistance: straddling the line between cost and benefit. Annu Rev Phytopathol **43:** 545–580

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, et al (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J **42:** 567–585

Crowe ML, Serizet C, Thareau V, Aubourg S, Rouze P, Hilson P, Beynon J, Weisbeek P, van Hummelen P, Reymond P, et al (2003) CATMA: a complete Arabidopsis GST database. Nucleic Acids Res **31:** 156–158

de Vrije T, Munnik T (1997) Activation of phospholipase D by calmodulin antagonists and mastoparan in carnation petals. J Exp Bot **48:** 1631–1637

Dickey AN, Faller R (2007) How alcohol chain-length and concentration modulate hydrogen bond formation in a lipid bilayer. Biophys J **92:** 2366–2376

Dong X (2004) NPR1, all things considered. Curr Opin Plant Biol **7:** 547–552

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol **42:** 185–209

Eulgem T (2006) Dissecting the WRKY web of plant defense regulators. PLoS Pathog **2:** e126

Forouhar F, Yang Y, Kumar D, Chen Y, Fridman E, Park SW, Chiang Y, Acton TB, Montelione GT, Pichersky E, et al (2005) Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. Proc Natl Acad Sci USA **102:** 1773–1778

Hallouin M, Ghelis T, Brault M, Bardat F, Cornel D, Miginiac E, Rona JP, Sotta B, Jeannette E (2002) Plasmalemma abscisic acid perception leads to RAB18 expression via phospholipase D activation in Arabidopsis suspension cells. Plant Physiol **130:** 265–272

Hirase A, Hamada T, Itoh TJ, Shimmen T, Sonobe S (2006) *n*-Butanol induces depolymerization of microtubules in vivo and in vitro. Plant Cell Physiol **47:** 1004–1009

Hilson P, Allemeersch J, Altmann T, Aubourg S, Avon A, Beynon J, Bhalerao RP, Bitton F, Caboche M, Cannoot B, et al (2004) Versatile gene-specific sequence tags for Arabidopsis functional genomics: transcript profiling and reverse genetics applications. Genome Res **14:** 2176–2189

Innes RW (2001) Mapping out the roles of MAP kinases in plant defense. Trends Plant Sci **6:** 392–394

Krinke O, Ruelland E, Valentová O, Vergnolle C, Renou JP, Taconnat L, Flemr M, Burketová L, Zachowski A (2007) Phosphatidylinositol 4-kinase activation is an early response to salicylic acid in Arabidopsis suspension cells. Plant Physiol **144:** 1347–1359

Kumar D, Gustafsson C, Klessig DF (2006) Validation of RNAi silencing specificity using synthetic genes: salicylic acid-binding protein 2 is required for innate immunity in plants. Plant J **45:** 863–868

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol **138:** 882–897

Lin C, Yu Y, Kadono T, Iwata M, Umemura K, Furuichi T, Kuse M, Isobe M, Yamamoto Y, Matsumoto H, et al (2005) Action of aluminum, novel TPC1-type channel inhibitor, against salicylate-induced and cold-shock-induced calcium influx in tobacco BY-2 cells. Biochem Biophys Res Commun **332:** 823–830

Lurin C, Andres C, Aubourg S, Bellaoui M, Bitton F, Bruyere C, Caboche M, Debast C, Gualberto J, Hoffmann B, et al (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16:** 2089–2103

Martinez C, Pons E, Prats G, Leon J (2004) Salicylic acid regulates flowering time and links defence responses and reproductive development. Plant J **37:** 209–217

Mateo A, Funck D, Muhlenbock P, Kular B, Mullineaux PM, Karpinski S (2006) Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. J Exp Bot **57:** 1795–1807

Munnik T, Arisz SA, De Vrije T, Musgrave AG (1995) Protein activation stimulates phospholipase D signaling in plants. Plant Cell **7:** 2197–2210

Overmyer K, Brosche M, Kangasjarvi J (2003) Reactive oxygen species and hormonal control of cell death. Trends Plant Sci **8:** 335–342

Profotová B, Burketová L, Novotná Z, Martinec J, Valentová O (2006) Involvement of phospholipases C and D in early response to SAR and ISR inducers in *Brassica napus* plants. Plant Physiol Biochem **44:** 143–151

Ruelland E, Cantrel C, Gawer M, Kader JC, Zachowski A (2002) Activation of phospholipases C and D is an early response to a cold exposure in Arabidopsis suspension cells. Plant Physiol **130:** 999–1007

Sakurai T, Satou M, Akiyama K, Iida K, Seki M, Kuromori T, Ito T, Konagaya A, Toyoda T, Shinozaki K (2005) RARGE: a large-scale database of RIKEN Arabidopsis resources ranging from transcriptome to phenome. Nucleic Acids Res **33:** D647–D650

Scott IM, Clarke SM, Wood JE, Mur LA (2004) Salicylate accumulation inhibits growth at chilling temperature in Arabidopsis. Plant Physiol **135:** 1040–1049

Thibaud-Nissen F, Wu H, Richmond T, Redman JC, Johnson C, Green R, Arias J, Town CD (2006) Development of Arabidopsis whole-genome microarrays and their application to the discovery of binding sites for the TGA2 transcription factor in salicylic acid-treated plants. Plant J **47:** 152–162

Vergnolle C, Vaultier MN, Taconnat L, Renou JP, Kader JC, Zachowski A, Ruelland E (2005) The cold-induced early activation of phospholipase C and D pathways determines the response of two distinct clusters of genes in Arabidopsis cell suspensions. Plant Physiol **139:** 1217–1233

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog **2:** e123

Wang D, Weaver ND, Kesarwani M, Dong X (2005) Induction of protein secretory pathway is required for systemic acquired resistance. Science **308:** 1036–1040

Wang X (2004) Lipid signaling. Curr Opin Plant Biol **7:** 329–336

Xu QM, Cheng JS, Ge ZQ, Yuan YJ (2005) Antioxidant responses to oleic acid in two-liquid-phase suspension cultures of Taxus cuspidata. Appl Biochem Biotechnol **125:** 11–26

Xu X, Chen C, Fan B, Chen Z (2006) Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell **18:** 1310–1326

Yu D, Chen C, Chen Z (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression. Plant Cell **13:** 1527–1540

Zheng Z, Mosher SL, Fan B, Klessig DF, Chen Z (2007) Functional analysis of Arabidopsis WRKY25 transcription factor in plant defense against Pseudomonas syringae. BMC Plant Biol **7:** 2