Phosphatidylinositol 4-Kinase Activation Is an Early Response to Salicylic Acid in Arabidopsis Suspension Cells[1][W]

Ondřej Krinke*, Eric Ruelland, Olga Valentová, Chantal Vergnolle, Jean-Pierre Renou, Ludivine Taconnat, Matyáš Flemr, Lenka Burketová, and Alain Zachowski

Université Pierre et Marie Curie-Paris 6 and Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7180, Laboratoire de Physiologie Cellulaire et Moléculaire des Plantes, Ivry-sur-Seine F–94200, France (O.K., E.R., C.V., M.F., A.Z.); Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague 166 28, Czech Republic (O.K., O.V., M.F.); Unité Mixte de Recherche, Institut National de la Recherche Agronomique 1165, Centre National de la Recherche Scientifique 8114, Unité de Recherche en Génomique Végétale, Evry F–91057, France (J.-P.R., L.T.); and Institute of Experimental Botany, v.v.i., Academy of Sciences of the Czech Republic, Prague 165 02, Czech Republic (L.B.)

Salicylic acid (SA) has a central role in defense against pathogen attack. In addition, its role in such diverse processes as germination, flowering, senescence, and thermotolerance acquisition has been documented. However, little is known about the early signaling events triggered by SA. Using Arabidopsis (*Arabidopsis thaliana*) suspension cells as a model, it was possible to show by in vivo metabolic phospholipid labeling with $^{33}P_i$ that SA addition induced a rapid and early (in few minutes) decrease in a pool of phosphatidylinositol (PI). This decrease paralleled an increase in PI 4-phosphate and PI 4,5-bisphosphate. These changes could be inhibited by two different inhibitors of type III PI 4-kinases, phenylarsine oxide and 30 μM wortmannin; no inhibitory effect was seen with 1 μM wortmannin, a concentration inhibiting PI 3-kinases but not PI 4-kinases. We therefore undertook a study of the effects of wortmannin on SA-responsive transcriptomes. Using the Complete Arabidopsis Transcriptome MicroArray chip, we could identify 774 genes differentially expressed upon SA treatment. Strikingly, among these genes, the response to SA of 112 of them was inhibited by 30 μM wortmannin, but not by 1 μM wortmannin.

During their development, plants are submitted to various stresses, either abiotic or biotic. To cope with these stresses, plants can synthesize molecules that will trigger adaptive responses. Salicylic acid (SA) is one of those molecules. Its role is mainly established in response to biotic stresses, where it acts both locally and systemically (Bostock, 2005). SA is indeed a mediator of systemic acquired resistance. Exogenous treatment with salicylates induced pathogenesis-related (PR) protein synthesis and enhanced resistance to infections. Both these effects were abolished in transgenic plants where endogenous SA was removed by expression of a bacterial gene encoding SA hydroxylase (*NahG*; Bostock, 2005). However, SA is produced in other physiological situations, such as chilling (Scott et al., 2004), exposure to ozone (Rao et al., 2002), and UV-C stress (Yalpani et al., 1994). Some physiological processes are disturbed in *NahG* transgenic plants or in mutant plants defective in SA biosynthesis. SA was thus shown to play a role in programmed cell death (Overmyer et al., 2003) and in tolerance to heat (Larkindale et al., 2005), osmotic stress (Borsani et al., 2001), reactive oxygen species (ROS; Danon et al., 2005), and high light stress (Mateo et al., 2006). SA reduces plant reproductive fitness (Heidel et al., 2004), improves seed vigor under stress conditions (Rajjou et al., 2006), induces earlier flowering (Martinez et al., 2004), and enhances senescence (Buchanan-Wollaston et al., 2005). The role of SA in those processes may be through SA-induced changes in gene transcription or protein accumulation (Gruhler et al., 2005; Kliebenstein et al., 2006; Rajjou et al., 2006; Thibaud-Nissen et al., 2006).

Whereas SA could intervene in such diverse physiological situations, little is known about the signals it triggers. Some events downstream of SA have recently been reported. Several high-affinity SA-binding proteins have been identified in tobacco (*Nicotiana tabacum*; Forouhar et al., 2005). SA induces an increase of ROS, which may then serve as signaling mediators themselves (Mateo et al., 2006). SA-induced protein kinase

[1] This work was supported by the Czech Science Foundation (grant no. 203–05–0559), the Czech Ministry of Education (grant nos. LC06034 and MSM 6046137305), the Centre National de la Recherche Scientifique, Université Pierre et Marie Curie-Paris 6, the French Ministry of Foreign Affairs (grant to O.K.), and the European Union Erasmus programme (grant to M.F.).

* Corresponding author; e-mail ondrej.krinke@vscht.cz; fax 420–220–445–167.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Ondřej Krinke (ondrej.krinke@vscht.cz).

[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.107.100842

belonging to the mitogen-activated protein kinase family is rapidly activated by phosphorylation in tobacco and its activation was shown to be necessary for the phosphorylation of the transcription factor WRKY1 (Menke et al., 2005). A number of studies have been focused on the upstream regulatory sequences of the *PR1* gene necessary for its induction by SA. One indispensable regulatory element is a consensus sequence, TGACG, binding TGA proteins belonging to the bZIP transcription factor family (Thibaud-Nissen et al., 2006).

Phospholipid signaling is emerging as a major component of responsive pathways to many stresses and hormones in plants (Meijer and Munnik, 2003). Phosphatidylinositol (PI) 3-kinase and its product PI 3-P [PI(3)-P] are required for auxin-induced production of ROS both in Arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*) and for root gravitropism in maize (Joo et al., 2005). PI 3-kinase inhibitors wortmannin or LY294002 inhibited abscisic acid-induced ROS generation and stomatal closure in broad bean (*Vicia faba*; Park et al., 2003). In this context, we intended to investigate the involvement of phospholipid signals in response to SA. We used Arabidopsis suspension cells, a model where SA uptake (Clarke et al., 2005) and SA-triggered response at the protein level (Gruhler et al., 2005) are well documented. By metabolic radiolabeling of phospholipids, we could show that PI 4-kinase is rapidly and transiently activated in response to SA. Using a microarray chip, we studied the SA transcriptome and its changes induced by wortmannin, an inhibitor of PI 4-kinase. We identified 112 genes whose response to SA was inhibited by 30 μM wortmannin, but not by 1 μM wortmannin. We also report potential cis-elements, which may coordinate the changes in gene expression upon SA treatment.

RESULTS

Cell Suspension of Arabidopsis Is a Suitable Model to Study the SA Signaling Pathway

By viability staining with Trypan blue, we determined that up to 1 mM SA did not cause a significant increase in cell death over a 24-h treatment period (data not shown). We verified that the cell culture expressed early and late marker genes of the SA signaling pathway (i.e. *GST6* and *PR1*; Blanco et al., 2005), in response to 250 μM SA (Fig. 1). At this stage, our results indicated that Arabidopsis suspension cells responded to SA at the level of gene expression and could be used as a model to study SA signaling.

SA Affects the Level of Radioactive Phosphoinositides in Vivo

The involvement of phospholipid signaling in response to SA was checked by studying radiolabeled phospholipids. A typical time course of phospholipid metabolic labeling by $^{33}P_i$ is shown in Figure 2. For phospholipids in which a phosphate group is monoesterified (i.e. phosphatidic acid, PI phosphate [PIP], and PI 4,5-bisP [PI(4,5)-P_2]), labeling was very fast, reaching a maximum after a few minutes, followed by a pseudoplateau lasting about 2 h and a phase corresponding to the progressive loss of labeling (Fig. 2A). For structural phospholipids in which radioactive phosphate is engaged only in a phosphodiester bond (i.e. PI, phosphatidylcholine [PC], phosphatidylethanolamine, or phosphatidylglycerol), labeling was much slower (Fig. 2B).

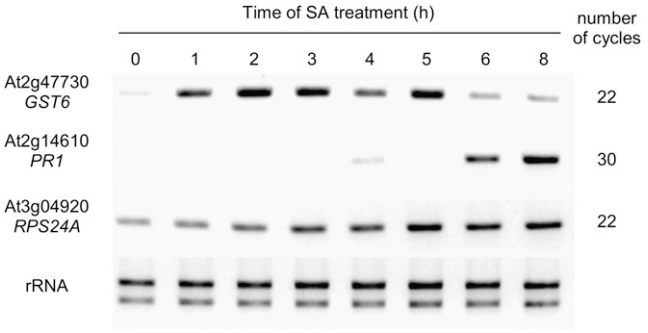

Figure 1. Expression of marker genes of the SA-responsive pathway in Arabidopsis cell suspensions. Time course of early marker gene *GST6* and late marker gene *PR1* expression was detected by RT-PCR after SA treatment of cells. The number of PCR cycles was optimized for each primer pair. 40S ribosomal protein S24 (*RPS24A*) was used as a housekeeping gene. GST6, Glutathione *S*-transferase6.

We chose to label cells with $^{33}P_i$ 5 min before lipid extraction because it allows good labeling of PI(4)-P and PI(4,5)-P_2. Cells were treated with 250 μM SA and lipids were extracted at different times after SA addition. Phospholipids were separated and the radioactivity incorporated into each lipid category was quantified. Labeling of PC was not affected by SA treatment; normalization was thus carried out by expressing the results as a labeled phospholipid to labeled PC ratio in SA-treated cells relative to this ratio in water-treated cells (Fig. 3). A sharp decrease in the relative PI to PC ratio was monitored between 15 and 90 min after SA application, followed by a temporary increase in this ratio (Fig. 3A). The decrease in PI to PC correlated to an increase in PIP to PC and PI(4,5)-P_2 to PC ratios. These changes in phosphoinositide ratio could be detected within 2 min after SA addition (Fig. 3B). Interestingly, these changes could also be seen when cells were labeled 15 min before lipid extraction, but could not be detected when labeled 24 h before lipid extraction (data not shown).

The changes in phosphoinositides were dose dependent and could be saturated at about 1 mM SA (Fig. 3C). Early decrease of labeled PI was not inhibited by cycloheximide, an inhibitor of protein synthesis, whereas late increase of labeled PI was inhibited and thus depended on protein synthesis (Supplemental Fig. S1).

SA-Induced PIP Is PI(4)-P

The fact that PIP and PI(4,5)-P_2 increased concomitantly makes a compelling argument for PIP being

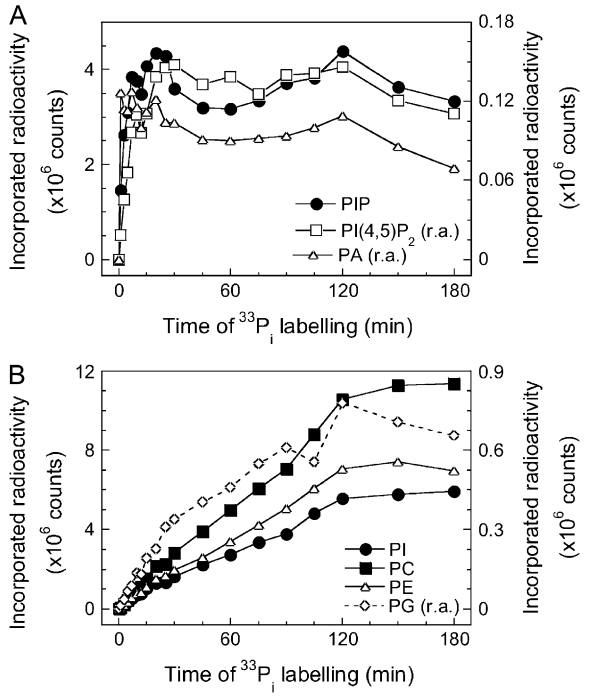

Figure 2. Time course of metabolic phospholipid labeling. $^{33}P_i$ was added to the cell suspension and lipids were extracted at the times indicated. Phosphomonoester (A) and phosphodiester (B) phospholipids show different labeling patterns. The experiment is representative of three independent repetitions. Results are expressed as counts incorporated into each lipid class. r.a., Right axis scale. PIP and PI(4,5)-P_2 were separated in the alkaline solvent system; other lipids were separated in the acid solvent system.

PI(4)-P. However, the alkaline solvent system routinely used to quantify PIP does not allow for separating PI(3)-P and PI(4)-P. To distinguish these two PIP isomers, a borate solvent system (Walsh et al., 1991) was used to develop thin-layer chromatography (TLC). SA-treated and water-treated cells were labeled with $^{33}P_i$ for 5 min before lipid extraction. Lipids were separated in the alkaline solvent system (Fig. 4A) and, in parallel, in the borate solvent system (Fig. 4B). The published retention factors (R_f) are 0.66 for PC, 0.46 for PI(4)-P, and 0.51 for PI(3)-P in the borate system. We found R_f values of 0.60 for PC and of 0.39 for PI(4)-P; the R_f value for the PI(3)-P spot would be expected to be close to 0.45, but no radioactive spot with this mobility was detected (Fig. 4B). Quantification of the PIP spots in both separation systems confirmed that more than 99% of the labeled PIP corresponded to PI(4)-P.

PI Decrease upon SA Treatment Is Catalyzed by a Type III PI 4-Kinase

Enzyme activities that could be responsible for the decrease of PI labeling and the increase in PI(4)-P and PI(4,5)-P_2 upon SA treatment are PI 4-kinases. There are two types of PI 4-kinases, which differ in their sensitivity to micromolar concentrations of wortmannin and by their Ca^{2+} dependence. No plant type II PI 4-kinase has been biochemically characterized yet. Based on animal and yeast (*Saccharomyces cerevisiae*) studies, type II PI 4-kinases are inhibited by high Ca^{2+} concentrations, but cannot be inhibited by micromolar concentrations of wortmannin, contrary to type III PI 4-kinases, which are inhibited by micromolar concentrations of wortmannin (Müller-Röber and Pical, 2002)

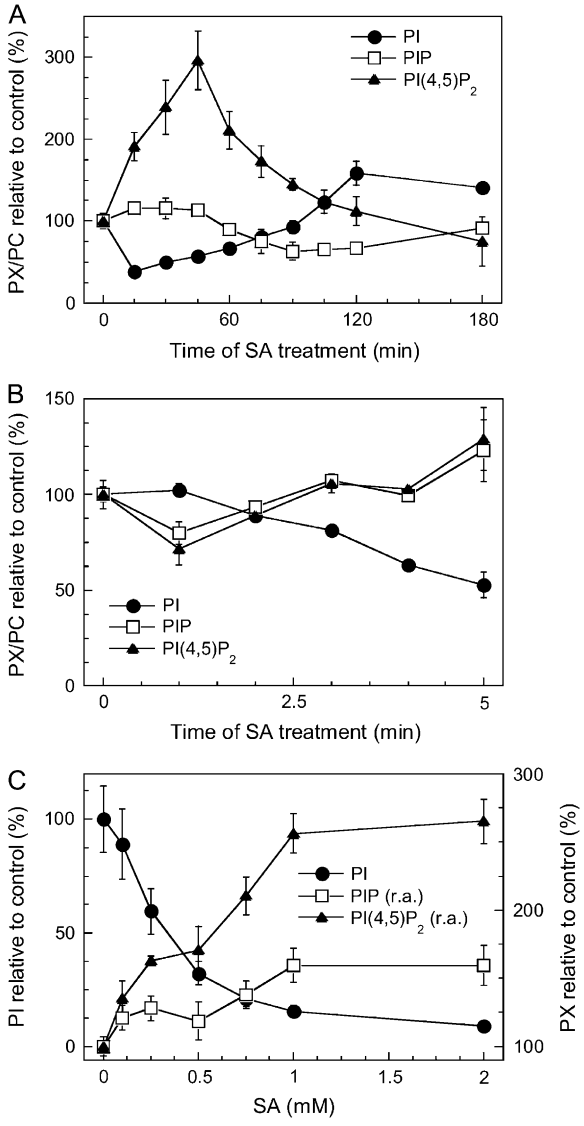

Figure 3. Labeled phosphoinositides in SA-treated Arabidopsis cells. A, Medium time course of phosphoinositide-labeling rate upon SA treatment. B, Changes in labeled phosphoinositide levels appear during the first minutes of SA treatment. C, Dose dependence of the observed changes. Cells were treated with the corresponding concentration of SA for 8 min before lipid extraction. r.a., Right axis scale. A to C, Cells were labeled with $^{33}P_i$ 5 min before lipid extraction. PIP and PI(4,5)-P_2 were separated in the alkaline solvent system; PI was separated in the acid solvent system. Values for water-treated cells were taken as controls (100%).

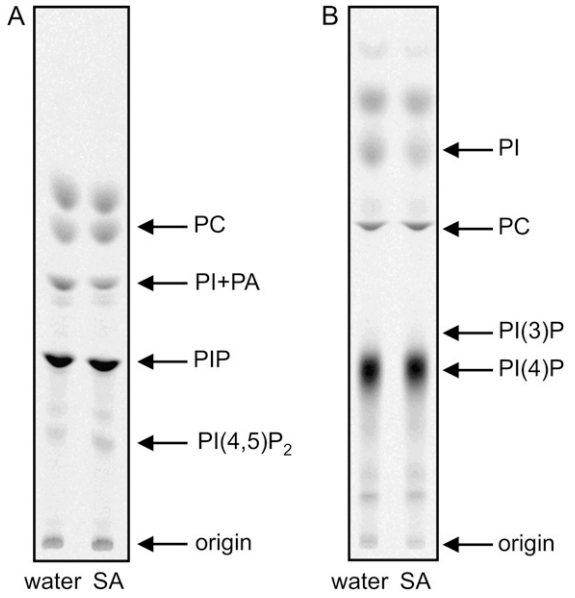

Figure 4. SA-induced PIP is PI(4)-P. Cells were treated with SA or with water 8 min before lipid extraction and labeled by $^{33}P_i$ 5 min before lipid extraction. Lipids were extracted and separated in parallel using an alkaline solvent system (A) and a borate solvent system (B). The expected position of PI(3)-P is also indicated (R_f = 0.45).

and phenylarsine oxide (PAO; Rajebhosale et al., 2003) and are not Ca^{2+} dependent (Müller-Röber and Pical, 2002). PAO and high concentrations of wortmannin inhibited the response (Fig. 5). These data are in favor of the PI decrease being due to the activity of a type III PI 4-kinase. Other PI kinases are active in plant cells. Plant PI 3-kinases are inhibited by submicromolar concentrations of wortmannin and by LY294002 (Turck et al., 2004). Low concentrations of wortmannin (up to 10 μM; Figure 5A) and 50 μM LY294002 (data not shown) did not affect the response to SA, confirming that PI 3-kinase is not responsible for the observed changes.

The increase in PI(4)-P and PI(4,5)-P_2 levels could be seen as a way of supplying substrates for the action of a phosphoinositide-specific phospholipase C (PI-PLC). PI-PLC inhibitors U73122 (60 μM) and edelfosine (150 μM) did not affect the increase of labeled PI(4,5)-P_2 (data not shown). Both these compounds were previously shown to inhibit PI-PLC in vivo in Arabidopsis cells (Vergnolle et al., 2005). Thus, the involvement of PI-PLC in the early SA response seems unlikely.

SA Induces Changes in the Arabidopsis Transcriptome

Preliminary results showed that SA-triggered transcript level changes were hardly recognized before 4 h, but became visible after 4 h. We therefore studied the changes of transcriptomes after 4 h of SA treatment. To do so, RNA samples from two independent biological repetitions were analyzed on the Complete Arabidopsis Transcriptome MicroArray (CATMA) chips as described in Lurin et al. (2004). RNA was extracted from 1 mg fresh weight of 5-d-old cells that had been treated for 4 h with water or SA. For each biological repetition, RNA samples for each condition were obtained by pooling RNA from three independent extractions. One dye swap (technical replicate with fluorochrome reversal) was made per biological repetition (i.e. four hybridizations per comparison). Differentially expressed genes were identified by a paired *t* test on the $\log_2$ ratios based on four hybridizations. The raw *P* values were adjusted by the Bonferroni method, which controls the family-wise error rate (FWER). With an FWER set at 5%, most of the probes (19,040 probes, i.e. 96.0% of total monitored probes), showed no changes in their transcript levels. Nevertheless, 475 genes (2.4% of total monitored genes) could be considered as up-regulated, whereas 317 (1.6%) were down-regulated. The list of the 792 differentially regulated genes is available as Supplemental Table S1.

The 25 most induced genes and the 25 most repressed genes are described in Tables I and II, respectively. Genes with unambiguous probe-to-gene assignment were extracted from the list of SA-regulated genes. They were then classified according to the Munich Information Center for Protein Sequences (MIPS) functional catalog categories (Ruepp et al., 2004), using the MIPS interface (Fig. 6). This database assigns function only for genes with sufficient supporting data. The most represented categories among the SA-induced genes were metabolism, cell rescue and defense, cellular transport, and protein synthesis and fate. For

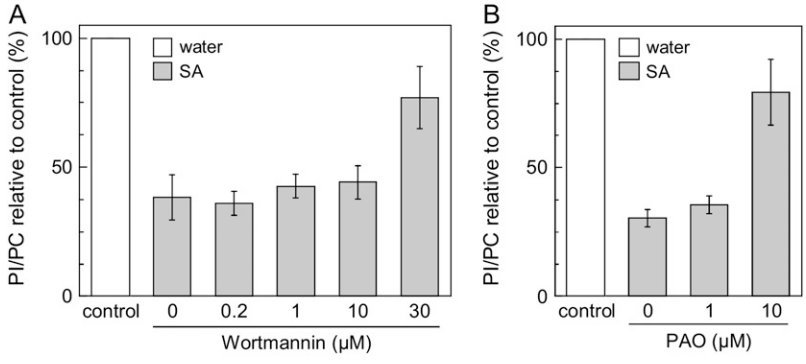

Figure 5. Inhibitory effect of wortmannin and PAO on the decrease in labeled PI caused by SA. Cells were incubated 45 min with wortmannin (A) or 15 min with PAO (B) prior to SA application and then treated with SA for 8 min before lipid extraction. Cells were labeled by $^{33}P_i$ 5 min before lipid extraction. Inhibitors were given in dimethylsulfoxide (final concentration 0.5% [v/v]). Controls (100%) represent water-treated cells. Results are averages of three independent repetitions.

Table I. *List of the 25 most induced genes after SA treatment, ranked by decreasing degree of induction*

AGI, Arabidopsis Genome Initiative gene index; $\log_2$ I, $\log_2$ average signal intensity (6–7 corresponds to a low level of expression, 14–15 corresponds to a high level of expression); $\log_2$ ratio, $\log_2 I_{SA} - \log_2 I_{water}$. Bonferroni-corrected *P* values were 0.05 in all cases.

Protein	Process	AGI	$\log_2 I_{water}$	$\log_2 I_{SA}$	$\log_2$ Ratio
Legume lectin family protein	Unknown	At5g03350	7.72	13.03	5.31
Amino acid transporter family protein	Amino acid transport	At4g35180	7.49	12.76	5.28
Expressed protein	Defense response	At1g31580	7.86	13.02	5.16
NPR1/NIM1-interacting protein 2 (NIMIN-2)	Unknown	At3g25882	7.50	11.79	4.29
WRKY family transcription factor	Regulation of transcription, DNA dependent	At5g22570	7.56	11.78	4.22
Expressed protein	Unknown	At1g56580	9.20	13.16	3.96
UDP-glucoronosyl/UDP-glucosyl transferase family protein	Metabolism	At3g11340	7.32	11.08	3.75
WRKY family transcription factor	Regulation of transcription, DNA dependent	At1g80590	7.55	11.05	3.50
Glycerophosphoryl diester phosphodiesterase family protein	Glycerol metabolism	At1g66970	8.92	12.38	3.46
UDP-glucoronosyl/UDP-glucosyl transferase family protein	Metabolism	At2g43820	7.45	10.88	3.43
Glutaredoxin family protein	Electron transport	At1g03850	7.41	10.80	3.39
Expressed protein	Unknown	At3g29240	9.77	13.13	3.36
Expressed protein	Unknown	At1g19960	6.86	10.22	3.36
NPR1/NIM1-interacting protein 1 (NIMIN-1)	Unknown	At1g02450	6.82	10.12	3.31
Oxidoreductase, 2-oxoglutarate and Fe(II)-dependent oxygenase family protein	Flavonoid biosynthesis	At5g24530	7.59	10.77	3.18
DC1 domain-containing protein	Intracellular signaling cascade	At2g44370	8.48	11.60	3.12
GTP-binding protein, putative	Small GTPase-mediated signal transduction	At1g09180	8.16	11.10	2.94
Leu-rich repeat family protein	G-protein-coupled receptor protein signaling pathway	At1g49750	9.12	12.06	2.94
ADP-ribosylation factor, putative	N-terminal protein myristoylation	At1g70490	11.06	13.97	2.92
Glutaredoxin family protein	Electron transport	At1g28480	7.32	10.20	2.88
WRKY family transcription factor	Regulation of transcription, DNA dependent	At2g40750	6.80	9.55	2.76
Toll-interleukin-resistance (TIR) domain-containing protein	Defense response signaling pathway, resistance-gene dependent	At1g72930	7.51	10.23	2.72
D-3-Phosphoglycerate dehydrogenase (3-PGDH)	L-Ser biosynthesis	At1g17745	8.74	11.46	2.72
Expressed protein	Unknown	At4g34630	11.13	13.78	2.65
Glutathione *S*-transferase6 (GST6)	Defense response	At2g47730	9.86	12.43	2.57

SA-repressed genes, the most represented categories were metabolism, cell fate and differentiation, protein synthesis and fate, and cellular transport. The category protein synthesis and fate, being both among the most induced and the most repressed genes, prompted us to investigate whether SA initiates the unfolded protein response (UPR). Three microarray experiments on whole plants of Arabidopsis identified marker genes of the UPR (Kamauchi et al., 2005). A list of UPR marker genes was compiled and crossed with our set of genes differentially expressed upon SA treatment. Of the 90 induced UPR markers, 19 were induced by SA and, of the 131 repressed UPR markers, 15 were repressed by SA treatment in our experiment (Supplemental Table S2).

Additional information was obtained from the analysis based on MapMan software (Thimm et al., 2004), which runs with less stringent gene-to-function assignments than the MIPS functional catalog. It is based both on experimental data and sequence homology predictions of gene function. These assignments can be mapped to user-defined graphic schemes. All relevant figures generated with this software are supplied as Supplemental Figure S2.

Expression data for genes that are known to be involved in phospholipid signaling are summarized in Supplemental Table S3. Among the genes with a corresponding CATMA probe only *PI4Kγ4* was significantly induced by SA treatment.

Effect of Wortmannin on the SA-Regulated Transcriptome

Because we have shown that 30 μM wortmannin inhibited SA-triggered PI 4-kinase activation, whereas 1 μM wortmannin did not (Fig. 5), we wanted to check the effects of wortmannin at both these concentrations on the SA-responsive transcriptome. RNA was isolated from 5-d-old cells that had been submitted to treatment with SA in the presence of 30 μM wortmannin (hereafter, SAW30), treatment with SA in the presence of 1 μM wortmannin (hereafter, SAW1), treatment with

Table II. *List of the 25 most repressed genes after SA treatment, ranked by decreasing degree of repression*

AGI, Arabidopsis Genome Initiative gene index; $\log_2$ I, $\log_2$ average signal intensity (6–7 corresponds to a low level of expression, 14–15 corresponds to a high level of expression); $\log_2$ ratio, $\log_2 I_{SA} - \log_2 I_{water}$. Bonferroni-corrected *P* values were 0.05 in all cases.

Protein	Process	AGI	$\log_2 I_{water}$	$\log_2 I_{SA}$	$\log_2$ Ratio
Pectate lyase family protein	Unknown	At4g24780	13.83	10.82	−3.02
Multicopper oxidase type I family protein	Pollen germination	At1g76160	13.09	10.54	−2.55
Multicopper oxidase type I family protein	Pollen germination	At4g22010	10.79	8.42	−2.37
Pectate lyase family protein	Unknown	At1g04680	11.99	9.86	−2.12
Xyloglucan:xyloglucosyl transferase, putative/xyloglucan endotransglycosylase, putative/endoxyloglucan transferase, putative	Carbohydrate metabolism	At4g30290	10.42	8.32	−2.10
Multicopper oxidase, putative (SKU5)	Tip growth	At4g12420	11.08	8.99	−2.08
Endo-1,4-β-glucanase, putative/cellulase, putative	Carbohydrate metabolism	At1g64390	10.68	8.60	−2.08
Nodulin family protein	Unknown	At4g34950	12.72	10.71	−2.01
Expressed protein	Unknown	At1g29980	10.93	8.94	−2.00
Aspartyl protease family protein	Proteolysis and peptidolysis	At3g61820	11.68	9.75	−1.93
Leu-rich repeat transmembrane protein kinase, putative	Transmembrane receptor protein Tyr kinase signaling pathway	At2g41820	9.83	7.93	−1.90
Expressed protein	Transport	At4g27720	11.61	9.74	−1.87
Phosphate-responsive 1 family protein	Unknown	At5g51550	11.86	10.04	−1.82
β-Expansin, putative (EXPB3)	Cell elongation	At4g28250	13.66	11.84	−1.82
Tubulin β-8 chain (TUB8)	Response to cold	At5g23860	11.30	9.49	−1.81
Myosin heavy chain-related	Unknown	At4g03620	11.37	9.56	−1.81
Fasciclin-like arabinogalactan-protein (FLA2)	Cell adhesion	At4g12730	11.31	9.52	−1.79
Expansin, putative (EXP6)	Cell wall modification during cell expansion (sensu *Magnoliophyta*)	At2g28950	11.28	9.50	−1.78
Ovule development protein aintegumenta (ANT)	Regulation of transcription, DNA dependent	At4g37750	9.50	7.72	−1.78
Phosphate-responsive 1 family protein	Unknown	At2g17230	9.61	7.84	−1.77
Plastocyanin-like domain-containing protein	Electron transport	At5g15350	11.35	9.58	−1.77
Osmotin-like protein, putative	Response to pathogen	At2g28790	9.90	8.14	−1.76
sugar transporter family protein	Carbohydrate transport	At1g73220	11.93	10.19	−1.74
Pentatricopeptide repeat-containing protein	Unknown	At2g30780	8.79	7.06	−1.73
Expressed protein	Unknown	At4g33625	9.82	8.12	−1.71

30 μM wortmannin (hereafter, W30), treatment with 1 μM wortmannin (hereafter, W1), or treatment with 0.5% (v/v) methanol (hereafter, M). Methanol at 0.5% (v/v) final concentration was used as the solvent for wortmannin. Cells were pretreated with inhibitors for 15 min and then treated with SA for 4 more hours where indicated. RNA extracted from three independent repetitions was pooled and reverse transcribed in the presence of Cy3-dUTP or Cy5-dUTP to perform two-color hybridization with the CATMA chip. One dye swap was made for four combinations: SAW1 versus SA, SAW30 versus SAW1, W1 versus M, and W30 versus W1. This has been repeated with another pool of three independent RNA extracts for each condition, leading to biological replicates. W30 or SAW30 is always compared to W1 or SAW1, respectively, to ensure that the effect of 30 μM wortmannin is not due to an effect already present at 1 μM. Hereafter, a gene regulated by a W30-sensitive pathway will imply that its regulation is disturbed by 30 μM wortmannin and that the effect cannot be attributed to 1 μM wortmannin.

We were interested in the identification of genes whose response to SA is affected by W30 and not by W1. Among the 792 genes that were differentially regulated in response to SA, 774 produced good hybridization in all tested dye swaps. Four genes whose transcript levels were different between W1 and M were not considered because they may be regulated by a W1-sensitive pathway, even in non-SA-treated conditions (Fig. 7). Among the remaining 770 genes, 312 showed a transcript level difference between SAW30 and SAW1. These genes are likely to be regulated via a W30-sensitive pathway. Four genes that showed transcript level changes between SAW1 and SA were excluded because they may correspond to genes regulated via a W1-sensitive pathway. Among the remaining 308 genes that were specifically regulated by a W30-sensitive pathway, 206 genes showed lower transcript levels in SAW30 versus SAW1. Among these 206 genes, 99 also had lower transcript levels in W30 versus W1, arguing for an effect on the regulatory events controlling the basal levels of those transcripts. For these genes, the effect of SAW30 versus SAW1 could therefore be a consequence of a perturbation of basal transcript level regulation and not the SA response. These genes were excluded because they may represent considerable bias for cluster analysis. Among the 308 genes that were specifically regulated by a

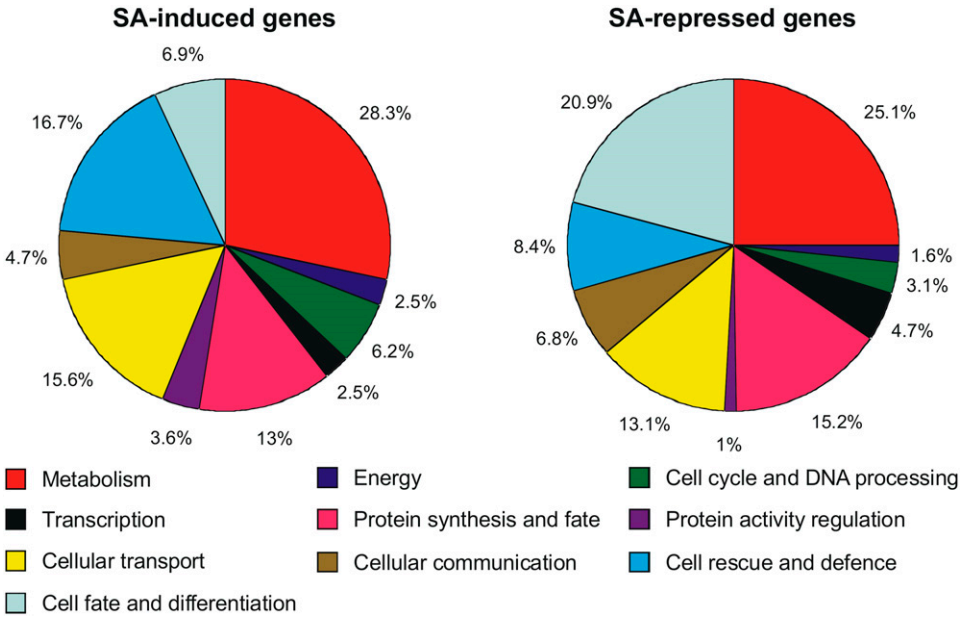

Figure 6. Functional categories of SA-induced and SA-repressed genes after 4 h of SA treatment in Arabidopsis suspension cells. Genes with unambiguous probe-to-gene assignment that were either up-regulated or down-regulated according to the microarray analysis were classified into functional categories using the MIPS functional catalog interface (Ruepp et al., 2004).

W30-sensitive pathway, 102 genes had higher transcript levels in SAW30 versus SAW1, but 80 of them showed the same changes in W30 versus W1. Again, for these genes, the effect of SAW30 versus SAW1 could be due to an effect on the regulatory events controlling the basal levels of those transcripts and not on the SA response, and thus these genes were also excluded. In the remaining group of 129 genes, which were specifically regulated by a W30-sensitive pathway during the SA response, 108 genes were SA induced and 21 were SA repressed. Among the 108 genes induced by SA, 99 showed lower transcript levels in SAW30 than in SAW1, showing an inhibitory effect on SA induction. Among the 21 SA-repressed genes, 13 showed higher transcript levels in SAW30 than in SAW1, showing an inhibitory effect on SA repression. Taken together, 112 of 129 genes (i.e. 87%) showed a repressing effect of 30 μM wortmannin on their response to SA treatment. Possibly underestimated, the number of genes induced by a W30-sensitive pathway, and not by a W1-sensitive one, represents 21% of SA-induced genes. The overall number of genes regulated by a W30-sensitive pathway represents 14% of all SA-regulated (i.e. repressed or induced) genes.

The results obtained by DNA microarray analysis were confirmed on a selection of seven genes showing high induction by SA and important inhibition of this response in the presence of 30 μM wortmannin. Reverse transcription (RT)-PCR analysis of an independent nonpooled set of RNAs revealed a clear and specific inhibitory effect of 30 μM wortmannin on SA induction for all tested genes (Supplemental Fig. S3).

To ascertain that it is reliable to assign a gene to a W30-sensitive pathway on both SA responsiveness and inhibitory effect in the W30 condition compared to the W1 condition, statistical analysis was performed. A cluster of 129 genes potentially regulated by W30 in response to SA (Fig. 7) was classified into four subgroups with respect to induction or repression of their expression by SA and inhibition or not of this response in the presence of 30 μM wortmannin compared to 1 μM. For each cluster, it was possible to calculate a theoretical number of genes based on the hypothesis that the transcript level difference between SAW30 and SAW1 was independent of the transcript level difference in the presence or absence of SA. The observed number of genes in each cluster was then compared to this theoretical number (Table III). It is clear that there was an overrepresentation of genes showing positive action of a W30-sensitive pathway on the response to SA (i.e. an inhibiting effect of W30 on the SA response) combined with the underrepresentation of genes not showing such a positive action. A Fisher's test indicated that these differences in distribution were not a product of random events ($P = 0.05$). Therefore, 112 (99 + 13) genes could be considered as responding to SA via the W30-sensitive pathway (Supplemental Table S4).

SA-Regulated Genes Revealed Common cis-Elements in Their Promoters

A subset of probes with unambiguous probe-to-gene assignment was extracted from the list of SA-regulated probes. Genes were divided into two groups: SA-induced genes and SA-repressed genes. The public repository of promoter sequences at The Arabidopsis Information Resource (TAIR; Rhee et al., 2003) was mined for common motifs in promoters (up to −1,000 bp) of each of these groups using the Motif Analysis tool. In parallel, these groups were analyzed using SIFT software (Hudson and Quail, 2003), where the promoter region spans by default up to −2,000 bp. Common motifs 4 to

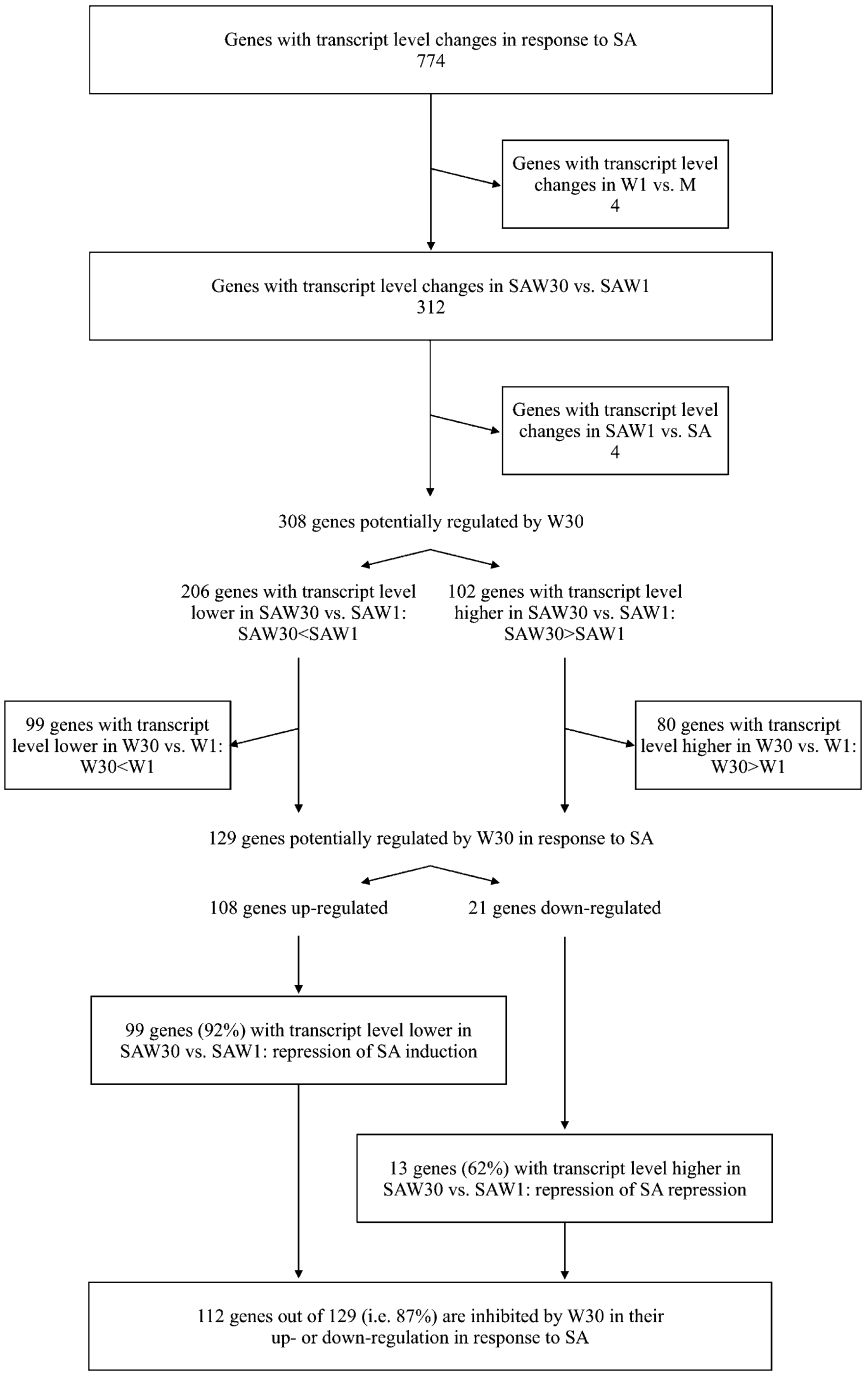

Figure 7. Determination of SA-regulated genes downstream of the PI 4-kinase pathway. Among the 3,270 genes that showed differential gene expression in one of the five tested comparisons, 129 showed transcript level changes both in SA versus water and in SAW30 versus SAW1 conditions. Additionally, for these genes, the transcript levels did not change in the same direction in W30 versus W1 as in SAW30 versus W1. Hence, these genes are potentially SA regulated via the PI 4-kinase pathway. Of these genes, 108 were up-regulated and 21 were down-regulated.

8 bp long were chosen for each group and separately statistically analyzed using R package software (version 2.4.1). Their frequency in promoters up to −1,000 bp on the whole-genome scale was determined by the Patmatch tool at TAIR and was compared to the observed number of appearances in the tested gene groups using a general bootstrapping method with 200 random trials. The probability that any random trial will produce the observed or higher/lower frequency of the given motif was assigned to each motif. Motifs were compared to the RIKEN Arabidopsis Genome Encyclopedia database using the built-in Promoter Search tool (Sakurai et al., 2005) and its name or the name of its closest homolog was assigned to it. We report several significantly overrepresented and underrepresented motifs specific both for SA-induced (Table IV) and SA-repressed genes (Table V).

We wanted to determine whether any of the motifs specific for SA-induced genes are overrepresented in the group of genes regulated by SA via a W30-sensitive

Table III. *Analysis of expected and observed clusters of genes classified by their response to SA and the effect of wortmannin on this response*

Expected number of genes for each cluster was calculated and compared to the actual number observed in our analysis. With respect to the expected numbers, the category printed in bold is overrepresented in the experimental data, whereas the categories written in italics are underrepresented. SA-induced and SA-repressed genes were considered separately. For each category, the differences in distribution correlated with the inhibitory effect of W30 versus W1 on the response to SA.

No. of Genes	SA > Water		Subtotal	SA < Water		Subtotal
	SAW30 > SAW1	SAW30 < SAW1		SAW30 > SAW1	SAW30 < SAW1	
Theoretical	*18*	**90**	108	**4**	*17*	21
Observed	*9*	**99**	108	**13**	*8*	21

pathway. For each motif, its frequency in the group of genes regulated by a W30-sensitive pathway was compared to its frequency in the subset of SA-induced genes using the same bootstrapping method on the subset of SA-induced genes. The same was done for the group of genes not regulated by a W30-sensitive pathway. Significantly over- and underrepresented motifs are listed in Table VI. One motif, the W-box (TTGACTT/TTGACY) motif, was found to be significantly overrepresented in the group of genes induced by SA via the W30-sensitive pathway and underrepresented in the group of genes induced by SA via the W30-insensitive pathway. These data suggest that WRKY transcription factors may be situated downstream of such W30-sensitive pathways in response to SA. Such analysis was not possible for SA-repressed genes because this subgroup of genes was too small (only 13 genes), making any statistics meaningless.

DISCUSSION

Together with works on SA uptake (Clarke et al., 2005) and SA-responsive proteomes (Gruhler et al., 2005) in cell suspensions, the fact that SA induced expression of genes known to be induced in Arabidopsis plants (Blanco et al., 2005; Kliebenstein et al., 2006; Thibaud-Nissen et al., 2006) in Arabidopsis cell suspensions allowed us to consider this system as a good model for studying SA signaling. The fact that cells have direct access to the medium assures synchronous uptake of radioactive label and applied agonist leading to high reproducibility of results (Meijer and Munnik, 2003), making cell suspensions the material of choice for studying plant lipid signaling.

Activation of a PI 4-Kinase as an Early Response to SA

In Arabidopsis suspension cells, phosphoinositide levels were studied after metabolic labeling with $^{33}P_i$. When cells are labeled for a very short time (5 or 15 min before lipid extraction), application of SA induces a rapid decrease in the pool of newly labeled PI, concomitantly with an increase of labeled PI(4)-P and P(4,5)-P_2. This makes a compelling argument for a PI 4-kinase being activated by SA. This is confirmed by the use of inhibitors: changes in labeled PI level are inhibited by PAO and high concentrations of wortmannin, indicating that these alterations are due to the activation of a type III PI 4-kinase, which is the prevalent PI 4-kinase activity in planta (Müller-Röber and Pical, 2002). Activation of PI 4-kinase occurs shortly after SA addition and lasts about 1 h, after which the activity returns to its initial level. Labeled PI(4,5)-P_2 follows the

Table IV. *cis-Elements identified in the promoters of SA-induced genes*

Analysis was performed on a subset of 450 genes with unambiguous probe-to-gene assignment. The number of appearances of each motif in promoters (−1,000 bp) of SA-induced genes (f_{obs}) was compared to its frequency in promoters of genes of the whole genome (f_{theor}) by a general bootstrapping technique. Only motifs significantly over- or underrepresented ($P < 0.05$) are listed.

Motif Description	Searched Motif	Found Motifs	f_{obs}	f_{theor}	Alternative	$P(f_{obs} = f_{theor})$
TATCCAY, GATA-box	TATCCA	368	0.82	0.59	Overrepresented	2.7e-10
I-box, MYB-like family	GATAAG	335	0.74	0.55	Overrepresented	3.3e-8
TAG1/RTBP1-binding, MYB-like family	AAACCC	270	0.60	0.82	Underrepresented	4.1e-5
W-box, WRKY family	TTGACY	736	1.64	1.22	Overrepresented	9.0e-15
W-box-like, WRKY family	TTGACTT	228	0.51	0.32	Overrepresented	2.7e-12
bZip	ACGT	2,470	5.49	4.38	Overrepresented	1.5e-11
A-box bZip variants	BACGTA	444	0.99	0.74	Overrepresented	5.3e-6
G-box bZip variants	YACGTG	372	0.83	0.60	Overrepresented	1.1e-5
C-box bZip variants	YACGTC	240	0.53	0.36	Overrepresented	8.9e-11
ASF-1, TGA family	TGACG	521	1.16	0.87	Overrepresented	3.2e-10
ASF-1-like, TGA family	TGACGTG	73	0.16	0.08	Overrepresented	1.2e-8
DRE/CRT core, DREB1A	ACCGACA	35	0.08	0.05	Overrepresented	2.9e-2
GT repeat	GTGTGTAT	37	0.08	0.06	Overrepresented	3.5e-2

Table V. *cis-Elements identified in the promoters of SA-repressed genes*

Analysis was performed on a subset of 304 genes with unambiguous probe-to-gene assignment. The number of appearances of each motif in promoters (−1,000 bp) of SA-repressed genes (f_{obs}) was compared to its frequency in promoters of genes of the whole genome (f_{theor}) by a general bootstrapping technique. Only motifs significantly over- or underrepresented ($P < 0.05$) are listed.

Motif Description	Searched Motif	Found Motifs	f_{obs}	f_{theor}	Alternative	$P(f_{obs} = f_{theor})$
TATCCAY, GATA-box	TATCCA	146	0.48	0.59	Underrepresented	1.5e-2
I-box, MYB-like family	GATAAG	120	0.39	0.55	Underrepresented	4.3e-4
TAG1/RTBP1-binding, MYB-like family	AAACCC	213	0.70	0.82	Underrepresented	4.0e-2
W-box, WRKY family	TTGACY	317	1.04	1.22	Underrepresented	7.6e-3
bZip	ACGT	1,450	4.77	4.38	Overrepresented	1.7e-2
A-box bZip variants	BACGTA	279	0.92	0.74	Overrepresented	2.2e-3
G-region-like (ethylene responsive)	AGCCGTT	41	0.13	0.06	Overrepresented	8.8e-7
DRE/CRT core, DREB1A	ACCGACA	40	0.13	0.05	Overrepresented	2.9e-9
GT repeat	GTGTGTAT	27	0.09	0.06	Overrepresented	2.1e-2

trends of labeled PI(4)-P both in SA dose dependence and time course. This suggests that PI(4,5)-P_2 accumulation is a simple consequence of PI(4)-P accumulation and that no supplemental control at the level of PIP 5-kinase has to take place during the response to SA. This is also the case in yeast, where regulation of PI(4,5)-P_2 synthesis rate takes place at the PI 4-kinase level (Godi et al., 1999). Besides this wortmannin-sensitive PI 4-kinase activation, the increase in PI(4,5)-P_2 could also result from inactivation of a phosphoinositide phosphatase. In Arabidopsis, the mutation of *suppressor of actin mutation domain phosphoinositide phosphatase-like9* and *fragile fiber3* (a gene encoding a type II inositol polyphosphate 5-phosphatase), has indeed led to the accumulation of PI(4,5)-P_2 in nonstressed plants (Zhong et al., 2004; Williams et al., 2005).

Increased synthesis of PI(4,5)-P_2 could be seen as an enhanced supply of substrate for PI-PLC activity, which would then produce inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. No production of $InsP_3$ was observed during the early stages of SA treatment (O. Krinke, unpublished data). This suggests a direct role of PI(4)-P and/or PI(4,5)-P_2 in response to SA. Based on the sequence of the Arabidopsis genome, van Leeuwen et al. (2004) identified 53 different proteins with pleckstrin homology domains, which can bind both PI(4)-P and PI(4,5)-P_2, and we have identified (O. Krinke, unpublished data) 84 proteins with a C2 domain, which can bind PI(4,5)-P_2 under low Ca^{2+} concentrations (Meijer and Munnik, 2003). All these proteins represent possible cellular targets of elevated PI(4)-P and/or PI(4,5)-P_2 levels.

It has to be underlined that the decrease of labeled PI upon SA treatment was only detected when cells were labeled for a very short time (5 or 15 min). During the short labeling time, only newly synthesized PI are preferentially labeled and the radiolabeled PI are still near their site of synthesis. This suggests that SA-triggered PI 4-kinase is located close to the site of PI synthesis, most probably on the endoplasmic reticulum (Sandelius and Morré, 1987). Interestingly, Arabidopsis PI 4-kinase α1 fused with GFP was visualized in endoplasmic reticulum membranes at the perinuclear region in insect cells (Stevenson-Paulik et al., 2003).

SA-Regulated Transcriptome

With more than 790 genes identified as SA regulated, this work is a very large collection of early SA-regulated plant genes. Transcriptomic response to SA and its physiological significance has already been discussed (Bostock, 2005; Thibaud-Nissen et al., 2006). Our data reveal several new aspects of the mode of action of SA: triggering of the UPR, modes of cellular redox regulation, and interaction with hormone-responsive pathways.

Table VI. *cis-Elements identified in the promoters of the subgroup of genes induced by SA via a W30-sensitive pathway*

Analysis was performed on subsets of genes with unambiguous probe-to-gene assignments. The number of appearances of each motif in promoters (−1,000 bp) of the cluster of 95 genes induced by SA via a W30-sensitive pathway ($f_{obs,1}$) was compared to its frequency in promoters of 450 genes induced by SA (f_{theor}) by a general bootstrapping technique. The same was done for 248 genes induced by SA via a W30-insensitive pathway ($f_{obs,2}$). Only motifs significantly over- or underrepresented in one of the tested clusters ($P < 0.05$) are listed. n.s.d., Observed frequency is not significantly different from the theoretical one ($P \geq 0.05$).

Motif Description	Searched Motif	f_{theor}	$f_{obs,1}$	$P(f_{obs,1} = f_{theor})$	$f_{obs,2}$	$P(f_{obs,2} = f_{theor})$
W-box, WRKY family	TTGACY	1.64	1.88	2.1e-2	n.s.d.	–
W-box-like, WRKY family	TTGACTT	0.51	0.73	3.0e-4	0.44	1.5e-2
Similar to LTRE	RGGCCY	0.64	n.s.d.	–	0.74	3.4e-2

An important overlap between SA and UPR transcriptomes was observed. UPR is triggered when stress, including biotic stress, causes protein folding in the ER to be slowed down, resulting in the temporary presence of a lot of unfolded proteins in the ER (Vitale and Ceriotti, 2004). It is characterized by induction of chaperones and proteins involved in disulfide bond formation and protein degradation. The observed transcriptome overlap includes protein disulfide isomerases and a luminal binding protein (BiP-3), which is an important marker of the UPR. Moreover, SA induced several components of the proteasome complex, especially F-box proteins, RING proteins, and members of ubiquitin-dependent protein catabolism. This further documents strong correlation between SA and UPR transcriptomes (Kamauchi et al., 2005). It has to be noted that elevation of PI(4,5)-P_2 level, which we monitored in response to SA, has also been reported during UPR in plants (Shank et al., 2001).

After 4 h of SA treatment, the ratio of reduced to oxidized glutathione is elevated in our cell suspensions (M. Flemr, unpublished data). More reducing environment, together with induction of thioredoxins and glutaredoxins, would lead to structural changes of several transcription factors (e.g. NPR1 and TGA1), which are known to be active in their reduced form (Bostock, 2005). Besides, induction of a number of glutathione *S*-transferases suggests mobilization of the detoxification machinery against oxidized molecules (Gruhler et al., 2005). SA acts in number of physiological situations and one of its modes of action could indeed be mobilization of antioxidant cell capacity against ROS produced during various stresses (Borsani et al., 2001; Rao et al., 2002; Danon et al., 2005; Mateo et al., 2006).

The biosynthetic pathway of ethylene was induced (e.g. a homolog of the tomato [*Solanum lycopersicum*] ethylene synthesis regulatory protein E8 and aminocyclopropane carboxylic acid oxidase) along with developmental genes related to ethylene signaling (ERF1 and members of the AP2/EREBP transcription factor family). The ethylene pathway was shown to be important for PR protein accumulation (Bostock, 2005). An overlap of SA and auxin response is also suggested: Two auxin efflux proteins were induced while an auxin influx protein (AUX1) was suppressed. This should result in reduced auxin uptake by the cells.

Effects of Wortmannin on the SA-Responsive Transcriptome

Because wortmannin at 30 μM, but not at 1 μM, inhibited SA-triggered PI 4-kinase activation, we studied the effects of wortmannin on the SA transcriptome at both concentrations. Treatments with 30 μM wortmannin (W30) were always compared to treatments with 1 μM wortmannin (W1) to exclude the effect already present at 1 μM. In our microarray analysis, we did not consider genes for which the differential response in the presence of 30 μM wortmannin was the same either with or without SA. This ensured that the observed effect of wortmannin was on the SA-triggered pathway and not on the cellular events already at play in unchallenged cells. In this way, we were able to identify 112 genes (mostly SA induced) whose response to SA is inhibited by 30 μM, but not by 1 μM wortmannin. For these genes, there is good correlation between the effect of wortmannin on SA-triggered PI 4-kinase activation and on the SA transcriptome. The addition of W30 mainly leads to inhibition of an SA response showing that the W30-sensitive pathway is implicated in the generation of the SA response and not in turning it off. Interestingly, the cluster of W30-sensitive genes contains transcription factors and PR genes important for systemic acquired resistance development (e.g. *NIMIN1*, *WRKY38*, several ERF/AP2 family transcription factors, TIR-class disease resistance proteins, various lectins, and class IV chitinase) and redox potential-regulating and regulated enzymes.

We have shown that wortmannin at 30 μM inhibited SA-triggered PI 4-kinase activity, whereas 1 μM wortmannin did not. It is therefore tempting to conclude that the genes, whose response to SA is inhibited by 30 μM but not by 1 μM wortmannin, are responding via a PI 4-kinase-dependent pathway. However rigorously, we cannot exclude that the W30 versus W1 effect on SA transcriptome is due to another mechanism. Indeed, wortmannin has been reported to inhibit PIP 5-kinase (Jung et al., 2002) or some protein kinases, such as ataxia-telangiectasia-mutated protein (Sarkaria et al., 1998). We cannot exclude that such protein kinases are upstream of some of the identified genes in response to SA.

MATERIALS AND METHODS

Cell Cultures

Cell suspensions of Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 and their maintenance are described in Vergnolle et al. (2005). Experiments were performed on 5-d-old cultures, which corresponds 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 by 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. Structural phospholipids and phosphatidic acid were separated in the acid solvent system composed of chloroform:acetone:acetic acid:methanol:water (10:4:2:2:1 [v/v/v/v]) according to Ruelland et al. (2002). Phosphoinositides were separated in the alkaline solvent system composed of chloroform: methanol:ammonia solution (5% [w/v]; 9:7:2 [v/v/v]), where the TLC plates were soaked in potassium oxalate solution before heat activation according to Ruelland et al. (2002). PI(3)-P and PI(4)-P were separated in the borate solvent system composed of methanol:chloroform:pyridine:boric acid:water:formic acid (88% [v/v]):2,6-di-*tert*-butyl-4-methylphenol:ethoxyquin (75:60:45:12: 7.5:3:0.375:0.075 [v/v/v/w/v/v/w/v]), where the TLC plates were soaked in trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid solution before heat activation according to Walsh et al. (1991). 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 Semiquantitative RT-PCR

Cells (7 mL of cell suspension; 1 g fresh weight) were filtered and immediately frozen in liquid nitrogen. RNA was extracted using phenol-chloroform extraction described in Vergnolle et al. (2005). For semiquantitative RT-PCR, 1 μg of total RNA was treated by DNase I from 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 were amplified in the successive 20-μL PCR with gene-specific primer pairs. The gene encoding a 40S ribosomal protein S24 (At3g04920) was used as a housekeeping gene. A suitable number of PCR cycles were used for each primer pair.

Transcriptome Studies

Microarray analysis was carried out at the Unité de Recherche en Génomique Végétale (Evry, France), 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 RNAs 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 (Perkin-Elmer-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. Statistical analysis was based on two dye swaps (i.e. four arrays, each containing 24,576 GSTs and 384 controls) as described in Lurin et al. (2004). Controls were used for assessing the quality of the hybridizations, but were not included in the statistical tests or the graphic representation of the results. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 (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 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. We considered as being differentially expressed the genes with an FWER $< 5\%$.

Data Deposition

Microarray data from this article were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE7495) and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: RS05-04_salicylic-acid) according to the Minimum Information About a Microarray Experiment standards.

Supplemental Data

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

Supplemental Figure S1. The early decrease of labeled PI is not inhibited by cycloheximide.

Supplemental Figure S2. Summary of interesting outcomes from the MapMan analysis.

Supplemental Figure S3. Confirmation of microarray results by RT-PCR analysis using an independent set of nonpooled RNAs.

Supplemental Table S1. List of genes differentially expressed upon SA treatment.

Supplemental Table S2. Overlap between SA-regulated and unfolded protein response-regulated transcriptomes.

Supplemental Table S3. Expression profiles of genes involved in phospholipid signaling.

Supplemental Table S4. Expression profiles of genes regulated by a W30-sensitive pathway in response to SA.

ACKNOWLEDGMENTS

We thank Marie-Laure Martin-Magniette (Unité de Recherche en Génomique Végétale, Institut National de la Recherche Agronomique, Unité Mixte de Recherche 1165, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8114, Evry, France) for help with the design of the microarray experiments, Françoise Thibaud-Nissen (The Institute for Genomic Research, Rockville, MD) for kindly providing expression data for SA-treated plants of Arabidopsis, and Radek Zíka (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Czech Republic) for help with the promoter analysis evaluation. We are grateful to Dr. Michael Hodges (Institut de Biotechnologie des Plantes, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8618, Université Paris-Sud 11, Orsay, France) for careful reading of the manuscript.

Received April 10, 2007; accepted May 7, 2007; published May 11, 2007.

LITERATURE CITED

Blanco F, Garreton V, Frey N, Dominguez C, Perez-Acle T, van der Straeten D, Jordana X, Holuigue L (2005) Identification of NPR1-dependent and independent genes early induced by salicylic acid treatment in Arabidopsis. Plant Mol Biol **59:** 927–944

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

Clarke A, Mur LA, Darby RM, Kenton P (2005) Harpin modulates the accumulation of salicylic acid by Arabidopsis cells via apoplastic alkalization. J Exp Bot **56:** 3129–3136

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

Danon A, Miersch O, Felix G, Camp RG, Apel K (2005) Concurrent activation of cell death-regulating signaling pathways by singlet oxygen in *Arabidopsis thaliana*. Plant J **41:** 68–80

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

Godi A, Pertile P, Meyers R, Marra P, di Tullio G, Iurisci C, Luini A, Corda D, de Matteis MA (1999) ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P_2 on the Golgi complex. Nat Cell Biol **1:** 280–287

Gruhler A, Schulze WX, Matthiesen R, Mann M, Jensen ON (2005) Stable isotope labeling of *Arabidopsis thaliana* cells and quantitative proteomics by mass spectrometry. Mol Cell Proteomics **4:** 1697–1709

Heidel AJ, Clarke JD, Antonovics J, Dong X (2004) Fitness costs of mutations affecting the systemic acquired resistance pathway in *Arabidopsis thaliana*. Genetics **168:** 2197–2206

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

Hudson ME, Quail PH (2003) Identification of promoter motifs involved in the network of phytochrome A-regulated gene expression by combined analysis of genomic sequence and microarray data. Plant Physiol **133:** 1605–1616

Joo JH, Yoo HJ, Hwang I, Lee JS, Nam KH, Bae YS (2005) Auxin-induced reactive oxygen species production requires the activation of phosphatidylinositol 3-kinase. FEBS Lett **579:** 1243–1248

Jung JY, Kim YW, Kwak JM, Hwang JU, Young J, Schroeder JI, Hwang I, Lee Y (2002) Phosphatidylinositol 3- and 4-phosphate are required for normal stomatal movements. Plant Cell **14:** 2399–2412

Kamauchi S, Nakatani H, Nakano C, Urade R (2005) Gene expression in response to endoplasmic reticulum stress in *Arabidopsis thaliana*. FEBS J **272:** 3461–3476

Kliebenstein DJ, West MA, van Leeuwen H, Kim K, Doerge RW, Michelmore RW, St Clair DA (2006) Genomic survey of gene expression diversity in *Arabidopsis thaliana*. Genetics **172:** 1179–1189

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

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

Meijer HJ, Munnik T (2003) Phospholipid-based signaling in plants. Annu Rev Plant Biol **54:** 265–306

Menke FL, Kang HG, Chen Z, Park JM, Kumar D, Klessig DF (2005) Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. Mol Plant Microbe Interact **18:** 1027–1034

Müller-Röber B, Pical C (2002) Inositol phospholipid metabolism in Arabidopsis: characterized and putative isoforms of inositol phospholipid kinase and phosphoinositide-specific phospholipase C. Plant Physiol **130:** 22–46

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

Park KY, Jung JY, Park J, Hwang JU, Kim YW, Hwang I, Lee Y (2003) A role for phosphatidylinositol 3-phosphate in abscisic acid-induced reactive oxygen species generation in guard cells. Plant Physiol **132:** 92–98

Rajebhosale M, Greenwood S, Vidugiriene J, Jeromin A, Hilfiker S (2003) Phosphatidylinositol 4-OH kinase is a downstream target of neuronal calcium sensor-1 in enhancing exocytosis in neuroendocrine cells. J Biol Chem **278:** 6075–6084

Rajjou L, Belghazi M, Huguet R, Robin C, Moreau A, Job C, Job D (2006) Proteomic investigation of the effect of salicylic acid on Arabidopsis seed germination and establishment of early defense mechanisms. Plant Physiol **141:** 910–923

Rao MV, Lee HI, Davis KR (2002) Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. Plant J **32:** 447–456

Rhee SY, Beavis W, Berardini TZ, Chen G, Dixon D, Doyle A, Garcia-Hernandez M, Huala E, Lander G, Montoya M, et al (2003) The Arabidopsis Information Resource (TAIR): a model organism database providing a centralized, curated gateway to Arabidopsis biology, research materials and community. Nucleic Acids Res **31:** 224–228

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

Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Guldener U, Mannhaupt G, Munsterkotter M, et al (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Res **32:** 5539–5545

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

Sandelius AS, Morré DJ (1987) Characteristics of a phosphatidylinositol exchange activity of soybean microsomes. Plant Physiol **84:** 1022–1027

Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT (1998) Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res **58:** 4375–4382

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

Shank KJ, Su P, Brglez I, Boss WF, Dewey RE, Boston RS (2001) Induction of lipid metabolic enzymes during the endoplasmic reticulum stress response in plants. Plant Physiol **126:** 267–277

Stevenson-Paulik J, Love J, Boss WF (2003) Differential regulation of two Arabidopsis type III phosphatidylinositol 4-kinase isoforms: a regulatory role for the pleckstrin homology domain. Plant Physiol **132:** 1053–1064

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

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J **37:** 914–939

Turck F, Zilbermann F, Kozma SC, Thomas G, Nagy F (2004) Phytohormones participate in an S6 kinase signal transduction pathway in Arabidopsis. Plant Physiol **134:** 1527–1535

van Leeuwen W, Ökrész L, Bögre L, Munnik T (2004) Learning the lipid language of plant signalling. Trends Plant Sci **9:** 378–384

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

Vitale A, Ceriotti A (2004) Protein quality control mechanisms and protein storage in the endoplasmic reticulum: a conflict of interests? Plant Physiol **136:** 3420–3426

Walsh JP, Caldwell KK, Majerus PW (1991) Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate. Proc Natl Acad Sci USA **88:** 9184–9187

Williams ME, Torabinejad J, Cohick E, Parker K, Drake EJ, Thompson JE, Hortter M, Dewald DB (2005) Mutations in the Arabidopsis phosphoinositide phosphatase gene *SAC9* lead to overaccumulation of PtdIns(4,5)P_2 and constitutive expression of the stress-response pathway. Plant Physiol **138:** 686–700

Yalpani N, Enyedi AJ, Leon J, Raskin I (1994) UV light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta **193:** 372–376

Zhong R, Burk DH, Morrison WH III, Ye ZH (2004) *FRAGILE FIBER3,* an Arabidopsis gene encoding a type II inositol polyphosphate 5-phosphatase, is required for secondary wall synthesis and actin organization in fiber cells. Plant Cell **16:** 3242–3259