Early genomic responses to salicylic acid in Arabidopsis

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Abstract Salicylic acid (SA) is a stress-induced hormone involved in the activation of defense genes. Here we analyzed the early genetic responses to SA of wild type and *npr1-1* mutant Arabidopsis seedlings, using Complete Arabidopsis Transcriptome MicroArray (CATMAv2) chip. We identified 217 genes rapidly induced by SA (early SAIGs); 193 by a NPR1-dependent and 24 by a NPR1-independent pathway. These two groups of genes also differed in their functional classification, expression profiles and over-representation of cis-elements, supporting differential pathways for their activation. Examination of the expression patterns for selected early SAIGs from both groups indicated that their activation by SA required TGA2/5/6 subclass of transcription factors. These genes were also activated by *Pseudomonas syringae* pv. tomato AvrRpm1, suggesting that they might play a role in defense against bacteria. This study gives a global idea of the early response to SA in Arabidopsis seedlings, expanding our knowledge about SA function in plant defense.

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Introduction

Salicylic acid (SA) is a key hormone of stress defense responses induced by biotrophic pathogens in plants (Alvarez 2000; Durrant and Dong 2004; Loake and Grant 2007). These responses allow plants not only to survive pathogen infection, but also to acquire a long-lasting systemic resistance (SAR, systemic acquired resistance) responsible for the protection from further infections by a broad range of pathogens (Grant and Lamb 2006). Several lines of evidence give support to the idea that SA plays a key role in the establishment of SAR. Tissue levels of SA and its glucosylated conjugates (SAG) increase locally and systemically after pathogen infection (Summermatter et al. 1995). Conversely, blockade of SA accumulation severely impairs the deployment of SAR (Gaffney et al. 1993; Delaney et al. 1994; Wildermuth et al. 2001). Further supporting a direct role of SA in pathogen-induced resistance, a series of experiments showed that treatment of plant tissues with exogenous SA or its functional analogs BTH (benzothiadiazole S-methylester) and INA (2,6-dichloroisonicotinic acid), was sufficient to trigger a defense reaction resembling SAR, that protects plants against a further pathogen infection (Cao et al. 1994; Lawton et al. 1996).

The resistance to infection triggered by SA seems to be mainly due to its ability to activate defense genes. This is supported by the fact that mutants in some components of the transcriptional machinery activated by SA, such as the co-activator NPR1 (*npr1* mutants) and the subclass II of TGA transcription factors (triple mutant *tga2-2/tga5-1/*

tga6-1), are severely impaired to deploy SAR, either in response to SA/INA treatment, or to avirulent pathogens (Cao et al. 1994; Zhang et al. 2003). Interestingly, analysis of the *tga2-2/tga5-1/tga6-1* triple mutant allowed the identification of TGA2, TGA5 and TGA6 as redundant factors involved in SA-mediated activation and basal repression of *PR1* gene (Zhang et al. 2003). Impairment in SAR development, although less severe than that of *npr1* and *tga2/tga5/tga6* mutants, was also recently reported in mutants in the SA-activated WRKY 18, one of the members of the large family of WRKY transcription factors activated by pathogens (Wang et al. 2006).

Serious efforts have been done to characterize the pathogen-induced transcriptome of Arabidopsis (reviewed in (Katagiri 2004; Eulgem 2005; Glazebrook 2005)). It is known that SA-induced genes (SAIGs) are an important part of this transcriptome, as revealed by the use of mutants in SA biosynthesis or signaling steps such as *sid2*, *npr1* or *nahG* plants (Maleck et al. 2000; Glazebrook et al. 2003; Tao et al. 2003; Eulgem et al. 2004; Katagiri 2004; AbuQamar et al. 2006). Studies aimed at identifying and characterizing SAIGs that are important for defense reaction have focused mainly on genes that depend on the coactivator NPR1 (NPR1-dependent SAIGs) (Maleck et al. 2000; Wang et al. 2005, 2006). Some of the best-characterized NPR1-dependent SAIGs so far are the pathogenesis-related genes (*PRs*), a group of genes that code for proteins with antimicrobial activity (van Loon et al. 2006). *PR-1*, a member of this group, is the most common marker for this defense pathway (Maleck et al. 2000; van Loon et al. 2006). Furthermore, SAIGs coding for endoplasmic reticulum-localized proteins associated to the protein secretory machinery and for WRKY transcription factors involved in *PR-1* activation, have been recently identified as primary targets of NPR1 involved in defense (Wang et al. 2005, 2006). Recently, two studies analyzing the response of Arabidopsis to short treatments with SA were published (Thibaud-Nissen et al. 2006; Krinke et al. 2007). One was performed in Arabidopsis cell suspensions and allowed the identification of SAIGs associated to the early activation of phosphatidylinositol 4-kinase (Krinke et al. 2007). The other study was performed in Arabidopsis plants and was oriented to identifying targets of TGA2 factor (Thibaud-Nissen et al. 2006).

Increasing evidence supports the idea that SA also plays a role in controlling the cellular redox balance at the onset of the SAR (Mou et al. 2003; Mateo et al. 2006; Holuigue et al. 2007). Treatment of Arabidopsis plants with INA produces a biphasic change of cellular redox potential (GSH/GSSG ratio): first a pro-oxidative effect and then an antioxidant effect of the SA analog (Mou et al. 2003). Associated to these changes, SA has been found to activate through a redox mechanism, NPR1, TGA1 (a member of

the TGA family of bZIP transcription factors) and the SA-responsive *as-1* promoter element (Garreton et al. 2002; Despres et al. 2003; Mou et al. 2003). In rice, SA has been proven essential for the protection of plants from oxidative stress caused by light, senescence, avirulent pathogens and treatment with methyl viologen (Yang et al. 2004). Consistently, in Arabidopsis, SA was required to restrict the oxidative stress and foster plant acclimation after transient exposure to high light intensity (Mateo et al. 2006). Interestingly, we and others found that SA uses a transient kinetics to activate genes coding for detoxifying or antioxidant enzymes such as glutathione *S*-transferases (GSTs) and glycosyltransferases (GTs) (Wagner et al. 2002; Lieberherr et al. 2003; Sappl et al. 2004; Uquillas et al. 2004; Blanco et al. 2005; Langlois-Meurinne et al. 2005). Besides activating stress defenses, SA is known to inhibit JA- and auxin-mediated responses, as part of the SA-mediated disease-resistance mechanism (Fujita et al. 2006; Ndamukong et al. 2007; Wang et al. 2007).

The aim of this study was to expand our knowledge about SA function in defense, identifying the subset of SAIGs that mediate early defense responses to SA in Arabidopsis seedlings (early SAIGs). For this purpose, we studied the expression profiles of wild type and *npr1-1* mutant Arabidopsis seedlings in response to short SA treatments, with the Complete Arabidopsis Transcriptome MicroArray (CATMAv2) chip. We identified 217 early SAIGs and found that a small proportion of them (24 genes) used an NPR1-independent pathway. Analysis of the functional categories of these genes indicates that NPR1-independent SAIGs mainly code for proteins involved in stress defense responses and C-compound metabolism, while proteins involved in protein phosphorylation and response to biotic stress were over-represented in the group of NPR1-dependent SAIGs. We confirmed, for a selection of these genes, that they are also responsive to inoculation with the avirulent bacterium *Pseudomonas syringae* pv. tomato AvrRpm1.

In silico promoter analysis of the early SAIGs identified in this study, and expression analysis performed for a selection of these genes (coding for glutaredoxins GRXC9 and GRXS13; the ANAC102 transcription factor, the unclassified SDRLP protein, a lectin like protein LLP and the NPR1-interactor NIMIN1), indicates that SA activates the transcription of these genes by a mechanism involving the subclass II of TGA transcription factors (TGA 2/5/6). Based on present findings and on previously published work, we propose a model to explain how a proper combination of SA-responsive promoter elements (*as-1-like*, TL-1 and TGA boxes), transcriptional regulators and co-regulators (TGA factors, NPR1 and other uncharacterized factors) could mediate the temporal control of early SAIGs expression.

Materials and methods

Plant growth conditions and treatments

The *Arabidopsis thaliana* wild-type, *npr1-1* mutant (Cao et al. 1994) and *tga6-1 tga2-1 tga5-1* triple mutant (Zhang et al. 2003) plants used in this study were from Columbia (Col-0) ecotype. Plants were grown in vitro in MS medium containing 15 g/l sucrose, under controlled conditions in a growth chamber (16 h light, 60 $\mu\text{moles m}^{-2} \text{s}^{-1}$, $22 \pm 2^\circ\text{C}$). Experiments were performed in 15 day-old seedlings (collected at 9:00 am) carefully taken from the MS medium and placed, root side down, in a Petri dish containing the corresponding solutions. For SA treatment, seedlings (wt and *npr1-1* mutant) were incubated in a 0.5 mM SA solution in MS medium, under continuous light (60 $\mu\text{moles m}^{-2} \text{s}^{-1}$) for 2.5 h in the case of microarray analysis, or for 0.5, 1, 2.5, 5, 8, 12 and 24 h for Northern blot analysis. Stock solution of 1 M SA (Sigma) was fresh-prepared in water. Control samples were incubated under the same conditions in MS medium.

To evaluate the need of de novo protein synthesis for SA-induced response, the protein synthesis inhibitor cycloheximide (CHX) was used. Seedlings were pre-incubated for 1 h in MS medium in the absence (control) or presence of 20 $\mu\text{g/ml}$ CHX and then exposed to 0.5 mM SA or MS for 2.5, 8 and 24 h. Stock solution of 20 mg/ml CHX (Sigma) was fresh-prepared in water. To evaluate transcript stability, seedlings were first incubated with 0.5 mM SA (prepared in MS) for 2 h and then transferred to MS medium (controls) or to a 0.5 mM SA solution, either in the presence or absence of 0.6 mM cordycepin (Sigma). A control sample treated with MS for 2 h was included. Samples were collected at 0.5, 1, 2, 3, 5 and 8 h.; whole seedlings were immediately frozen in liquid nitrogen and stored at -70°C until RNA purification.

Microarray analysis

Total RNA was extracted from control and SA-treated seedlings of wild type and *npr1-1* mutant plants (pool of 20 seedlings for replicate, two independent biological replicates for each treatment) with Trizol®, according to the manufacturer's instructions (Invitrogen). In each case, we compared SA-treated seedlings with that from control-treated seedlings on an Arabidopsis cDNA microarray, CATMAv2. Each comparison was repeated in dye-swap leading to 4 hybridizations per comparison.

The CATMAv2 array used in this study was an updated version of CATMAv1 array that was previously described and validated (Hilson et al. 2004; Allemeersch et al. 2005). This array consisted of 23,520 features, including 18,981 unique GSTs, 768 positive/negative controls (Amersham

BioSciences), and 243 blanks, printed on Type VIIstar reflective slides (Amersham BioSciences) using a Lucidea Array spotter (Amersham BioSciences). The annotation of the clone set can be accessed via the ArrayExpress database as accession number A-MEXP-10 (<http://www.ebi.ac.uk/arrayexpress>) or via the VIB MicroArray Facility Web site (<http://www.microarrays.be>). Prior to hybridization, the slides were washed in $2\times$ saline-sodium phosphate-EDTA buffer, 0.2% SDS for 30 min at 25°C . RNA was amplified using a modified protocol of in vitro transcription (Puskas et al. 2002). Briefly, 5 mg of total RNA was reverse transcribed to double-stranded cDNA using an anchored oligo(dT) 1 T7 promoter [5'-GGCCA GTGAATTGTAATACGACTCACTATAGGGAGGCGGT 24(ACG)-3' (Eurogentec, Seraing, Belgium)]. From this cDNA, RNA was produced via T7-in vitro transcriptase until an average yield of 10–30 mg of amplified RNA. The amplified RNA (5 mg) was labeled with dCTP-Cy3 or Cy5 (Amersham BioSciences) by reverse transcription using random nonamer primers (Genset, Paris). The resulting probes were purified with Qiaquick (Qiagen) and analyzed for amplification yield and incorporation efficiency by measuring the DNA concentration at 280 nm, Cy3 incorporation at 550 nm, and Cy5 incorporation at 650 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Rockland, DE). A good target had a labeling efficiency of 1 fluorochrome every 30–80 bases. For each target, 40 pmol of incorporated Cy5 or Cy3 were mixed in 210 μl of hybridization solution containing 50% formamide, $1\times$ hybridization buffer (Amersham BioSciences), 0.1% SDS. Each spike mix was hybridized against the reference RNA (spikes at 100 cpc) and repeated with dye swap to make up 14 hybridizations in total. Hybridization and post hybridization washing were performed at 45°C with an Automated Slide Processor (Amersham BioSciences). Post-hybridization washing was done in $1\times$ sodium chloride/sodium citrate buffer (SSC), 0.1% SDS, followed by $0.1\times$ SSC, 0.1% SDS and $0.1\times$ SSC. Arrays were scanned at 532 nm and 635 nm using a Generation III scanner (Amersham BioSciences). Images were analyzed with ArrayVision (Imaging Research, St. Catharines, Canada). All protocols are available at the VIB MicroArray Facility Web site (<http://www.microarrays.be>) and at ArrayExpress under accession numbers P-MEXP-578, P-MEXP-579, P-MEXP-581, P-MEXP-582 for Cy3 labeling, Cy5 labeling, hybridization, and scanning, respectively.

Microarray data analysis

Spot intensities were measured as artifact removed total intensities, subtracted with the local background (sARVol), and filtered based on two standard deviations above background. For each gene, ratios of red (Cy-5) over green

(Cy-3) intensities (I) were calculated and normalized via a Lowess Fit of the log₂ ratios [$\log_2(\text{Icy-5/Icy-3})$] over the log₂ total intensity [$\log_2(\text{Icy-5} \times \text{Icy-3})$]. Genes were considered differentially expressed if the normalized ratios were statistically significant using a two-tailed t test ($P < 0.01$) between the biological and dye-swap repeats and were higher or equal to two-fold.

Functional classification of genes was performed according to the Functional Catalogue (FunCat) version 2.1 available at <http://mips.gsf.de/proj/funcatDB> (Ruepp et al. 2004). Over-represented functional categories in the two lists of early SAIGs compared to the complete Arabidopsis genome were obtained according to the MIPS classification using BioMaps tool from the VirtualPlant webpage (<http://virtualplant.bio.nyu.edu>). Hypergeometric method and Bonferroni correction were used for the analysis with a P value cutoff of 0.05. To collect expression data of selected genes in response to stress, the stimulus analysis tool from the public GeneVestigator V3 database, which compile information of Affymetrix GeneChip experiments, was used (<https://www.genevestigator.ethz.ch/>; (Laule et al. 2006). Intersection analysis tool from VirtualPlant version 0.9 (<http://virtualplant-prod.bio.nyu.edu/cgi-bin/virtualplant.cgi>) was used to analyze the intersections between different lists of genes from microarray experiments.

Northern analysis

For Northern blot analysis, 10 µg total RNA (isolated by using Trizol®) of the different samples were separated on formaldehyde-agarose gels, as previously described (Blanco

et al. 2005). Radioactive signals were either quantified by a Phosphorimager (Cyclone, Storage Phosphor screen, Packard Bioscience Company) or detected by autoradiography after 12 h exposure. Gene-specific DNA probes were obtained by PCR using cDNA from SA-treated seedlings as template and specific primers designed according to each gene sequence. Amplified probes were cloned and sequenced. Sequences of the gene specific primers are described in Table 1. Probes were labeled by PCR as described (Blanco et al. 2005).

Plant inoculation with *P. syringae*

For bacterial inoculation assays, 6-week old plants grown on soil under controlled condition in a growth chamber ($22 \pm 2^\circ\text{C}$, 10 h light, $100 \mu\text{moles m}^{-2} \text{s}^{-1}$) were inoculated with *Pseudomonas syringae* pv. tomato strain DC3000 that expresses the AvrRpm1 gene (Pst/AvrRpm1). A bacterial suspension of 5×10^6 cfu/ml in 10 mM MgCl_2 was infiltrated on the abaxial side of the leaves with a 1 ml syringe (Pavet et al. 2006). Mock-treated plants were infiltrated with 10 mM MgCl_2 . Bacteria- and mock-infiltrated leaf samples were collected at 0, 5, 21, 26 and 48 h post infiltration, immediately frozen in liquid nitrogen and stored at -70°C until RNA purification or SA quantification.

Quantification of SA

Free and conjugated SA extracted from leaf tissues was quantified by HPLC as described (Verberne et al. 2002).

Table 1 Primers used to obtain gene-specific probes

AGI	Forward primer	Reverse primer
AT5G24530	GGTGAGTGTTCGCGGTGAGTTCTTTAGC	GGGCGTTTGGGTGCGGTATGAGCA
AT1G02450	TACAATTATTCCTAGAGACCAT	TAATAATCACAACGCTAACAAAT
AT3G25882	GTTCGGACGGTAACGGAGGAAGAGGT	GTTCAGATCCAAACCCAAACCCTGTAAAC
AT1G28480	GTGCAAGAAATTATAACATGACGACAAC	TTTACAATCACAACCACAGAGCCCCAACT
AT1G03850	GTCACCGTGGACGAAGACCG	CCAGTGGTGGTTGAGATTGG
AT4G13180	ATCGCGATTACCTCCACCTCC	AACGGCGGCCTTTGATGC
AT1G76680	GATCTGGCATGTTGGCCGCG	CGATCTCCTTAGCAACTGCATC
AT5G03350	TTGGGAAAATGAAACACTGGTC	CATTCCGGTTACAACCTTCTGATAC
AT5G22570	TCACCGGATCCGATTTACTACGAC	AGCGGCTTTCCTTCTCCTGA
AT5G64250	ATGCGAGAGGGATTGGTCTTCT	ATGGCGTCTACTCCTACAGCAACA
AT3G11340	GACGCTCTTTGGTACTTCACTCACGA	TACGGAATTTGTTGCTTGCTTGCTCTAA
AT1G37130	CCTAACGGCGGGCTCATGTCTC	TCTCGCATAATCGCCTGCGAA
AT3G53750	GCTATGTATGTCGCCATTCAAGC	CATCATATTCTGCCTTTGC(A/G)ATCC
AT2G14610	GTAGGTGCTCTTGTCTCTCC	CACATAATTCCCACGAGGATC
AT2G29420	TGATTCCGGTCTTGTTCATAATG	AACGCCACCAAAGTCGCCAC
AT5G63790	GGACTTTGCTCTCTTCTCC	GCACATCTCCGGCAAAG

Promoter analysis

Over-represented promoter elements were independently searched in the groups of 31 NPR1-independent and 197 NPR1-dependent SAIGs, using the Motif-Sampler software (<http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html>) (Thijs et al. 2002). For each group of genes, 1 kb of the genomic sequence upstream from the inferred translational start site was downloaded from TAIR database (*The Arabidopsis Information Resource*, <http://www.arabidopsis.org>). Promoters analysis was performed essentially as described (Blanco et al. 2005), except that the motif lengths (w) were set at 16 and 20 bp. For each search, the algorithm was iterated 2,000 times; independent outputs were merged and the 10 highest scored motifs were selected and ranked according to their log-likelihood scores. Finally, to search for known plant promoter elements that matched the selected motifs, PLACE database (Database of Plant Cis-acting Regulatory DNA elements, <http://www.dna.affrc.go.jp/PLACE/>) was used (Higo et al. 1999).

Results

Identification of early SA-induced genes, determination of NPR1 dependence and functional classification

To analyze the early genetic response of Arabidopsis to SA, transcript profiles of seedlings incubated for 2.5 h with or without SA were compared using CATMAv2 (Complete Arabidopsis Transcriptome MicroArray) DNA chips including 18,981 unique gene specific tags (GSTs) (Lurin et al. 2004). The requirement of NPR1 for this response to SA was determined comparing transcript profiles of wild type and *npr1-1* mutant seedlings. Genes differentially activated by SA were selected according to the results of a t-test analysis using a two-fold increase in expression as threshold. We selected 217 SAIGs (1.1% of total genes monitored) from wild type plants and 34 SAIGs (0.18%) from *npr1-1* mutant plants (see supplementary Table S1). Genes whose steady-state mRNA levels increased in response to SA exclusively in the wild type plants, were considered as “early NPR1-dependent SAIGs” (193 genes), while genes whose mRNA levels increased after SA treatment in both wild type and *npr1-1* plants were considered as “early NPR1-independent SAIGs” (24 genes). Genes that showed reduced responsiveness to SA in the *npr1-1* background compared with the wild type were included in the early NPR1-independent SAIGs.

In a previous study, we identified 14 early SAIGs (Uquillas et al. 2004; Blanco et al. 2005). In the CATMAv2 chip, only 6 of these genes were represented by probes and,

although 5 of them seemed to be induced by SA, the induction values were statistically significant for only 3 of these genes (At2g29420, At5g54610, At3g11280). These 3 genes were included in the list of 217 SAIGs. Therefore, for further classification and expression analysis of early SAIGs, we considered the 217 genes identified in this study and the 11 SAIGs (4 NPR1-dependent and 7 NPR1-independent) that were identified and corroborated previously (Uquillas et al. 2004; Blanco et al. 2005). The list of early NPR1-dependent SAIGs with activation levels higher than 3 (46 out of 197 genes) is shown in Table 2, whereas the list of the 31 early NPR1-independent SAIGs is shown in Table 3.

Early NPR1-dependent and NPR1-independent SAIGs were classified in functional categories according to the Munich Information Center for Protein Sequences classification (MIPS) (Ruepp et al. 2004), using the FunCat database. Over-represented MIPS categories were identified by using the BioMaps tool from Virtual Plant database (<http://virtualplant.bio.nyu.edu>). Different sets of functional categories were overrepresented in NPR1-dependent and NPR1-independent SAIGs (Table 4). In the group of 197 early NPR1-dependent SAIGs, the categories “Metabolism” and “Protein Fate” (represented by subcategories “phosphate metabolism” and “protein modification by phosphorylation”) and the categories “Cell rescue, Defense and Virulence”/“Interaction with the environment”/“Cell fate” (subcategories associated to defense responses to pathogens) were overrepresented (Table 4). Accordingly, the functional groups mainly represented in this class of SAIGs were protein kinases (36 genes), disease resistance proteins (11 genes) and WRKY transcription factors (7 genes) (see Table 2 and supplementary Table S1).

On the other hand, the over-represented categories in the 31 early NPR1-independent SAIGs were “Metabolism” (subcategory “C-compound and carbohydrate metabolism”) and “Cell Rescue, Defense and Virulence”/“Interaction with the environment” (subcategories associated to detoxification and response to chemicals and hormones) (Table 4). Consistently, 6 genes coding for UGTs and 4 genes coding for glutaredoxins and GSTs were identified in this class of SAIGs (Table 3).

To get a preliminary idea if the SAIGs identified in this study play a role in defense responses against different stressful stimuli, we analyzed the available transcriptomic expression data for these genes using GeneVestigator (<http://www.genevestigator.ethz.ch/>) (Laule et al. 2006). We did an independent search of the expression data for the list of early NPR1-dependent SAIGs that displayed an increase of more than 3 times in their steady-state mRNA levels in response to SA (listed in Table 2) and for the list of early NPR1-independent SAIGs (listed in Table 3). Early NPR1-independent SAIGs were more responsive

Table 2 NPR1-dependent SAIGs

AGI	Alias	SA wt	Annotation	FunCat	SubCategories	References
<i>Protein with binding function</i>						
At5g03350	<i>LLP</i>	191.3	Legume lectin family protein	C-compound binding		
At1g02450	<i>NIMIN-1</i>	55.7	NPR1/NIM1-interacting protein 1	Disease, virulence and defense; response to biotic stimulus; transcriptional control; protein binding		Weigel et al. (2005)
At3g25882	<i>NIMIN-2</i>	50.9	NPR1/NIM1-interacting protein 2, encodes a kinase that physically interacts with NPR1/NIM1	Unclassified protein		Weigel et al. (2005)
At5g54610	<i>ANK</i>	11.9	Ankyrin repeat family protein	Plant signaling molecules response; chemoperception and response; protein binding		Blanco et al. (2005)
At3g56710	<i>SIB1</i>	7.6	Sigma factor binding protein 1; interacts with Sig1R4	Protein binding; chloroplast		
At1g73800		5.0	Calmodulin-binding protein	Protein binding		
At2g46430	<i>CNGC4</i>	3.1	Cyclic nucleotide gated channel (CNGC4), downstream component of the signaling pathways leading to HR/resistance	Protein binding, cyclic nucleotide binding, ion channel		Gobert et al. (2006)
<i>Protein fate (protein modification and degradation)</i>						
At3g09010		7.0	Protein kinase family protein	Protein modification by phosphorylation-dephosphorylation		
At1g21250 ^a	<i>WAK1</i>	5.3	Cell wall-associated kinase	Plant signaling molecules response; chemoperception and response; receptor enzyme mediated signalling		He et al. (1998); Park et al. (2001); Blanco et al. (2005)
At1g34750		4.3	Protein phosphatase 2C, putative	Protein modification by phosphorylation-dephosphorylation		
At4g25110		4.2	Caspase family protein; similar to LOL3 (LSD ONE LIKE 3), caspase/cysteine-type endopeptidase	Protein/peptide degradation; apoptosis		Vercammen et al. (2004)
At1g51800		3.8	Leucine-rich repeat protein kinase, putative	Protein modification by phosphorylation-dephosphorylation		
At1g65800	<i>ARK2</i>	3.6	Armadillo repeat kinesin 2. Putative receptor-like serine/threonine protein kinases that is similar to brassica self-incompatibility (S) locus	Receptor enzyme mediated signalling; protein modification by phosphorylation-dephosphorylation		Sakai et al. (2008)
At1g16260		3.5	Protein kinase family protein. Identical to Wall-associated receptor kinase-like 8 precursor	Protein modification by phosphorylation-dephosphorylation		
At4g26070	<i>MEK1</i>	3.4	Member of MAP Kinase Kinase	Oxidative stress response; disease virulence and defense; chemoperception and response; response to biotic stimulus; protein kinase; protein binding		Xing et al. (2007)
At2g02800	<i>APK2b</i>	3.1	Protein kinase 2B	Protein modification by phosphorylation-dephosphorylation		Ito et al. (1997)

Table 2 continued

AGI	Alias	SA wt	Annotation	FunCat SubCategories	References
<i>Transcription</i>					
At5g22570	ATWRKY38	31.2	Member of WRKY Transcription Factor; Group III	Transcriptional control	Wang et al. (2006)
At5g01900	ATWRKY62	20.1	Member of WRKY Transcription Factor; Group III	Transcriptional control	Mao et al. (2007)
At2g40750	ATWRKY54	7.2	Member of WRKY Transcription Factor; Group III	Transcriptional control	Wang et al. (2006)
At4g23810	ATWRKY53	4.0	Member of WRKY Transcription Factor; Group III	Transcriptional control	Wang et al. (2006)
At4g31800	ATWRKY18	3.8	Member of WRKY Transcription Factor	Transcriptional control; disease, virulence and defense; chemoperception and response; response to biotic stimulus; plant signaling molecules response	Wang et al. (2006)
<i>Perception and response to stimuli (disease resistance proteins)</i>					
At3g04210		3.6	Disease resistance protein (TIR-NBS class), putative	Disease, virulence and defense; receptor enzyme mediated signalling; ATP binding	Consonni et al. (2006)
At1g11310	ATMLO2	3.4	Mildew resistance locus O 2	Disease virulence and defense; plant defense response; cell death; protein binding	
At4g26090	RPS2	3.2	Disease resistance protein RPS2 (CC-NBS-LRR class)	Disease virulence and defense; response to biotic stimulus; cell death; protein binding	Cai et al. (2006)
At5g36930		3.1	Disease resistance protein (TIR-NBS-LRR class), putative	Disease virulence and defense; receptor enzyme mediated signalling; ATP binding; mitochondrion	Orgil et al. (2007)
At3g50480		3.0	Homolog of RPW8 that confers broad-spectrum resistance to powdery mildew pathogens	Unclassified protein	
<i>Metabolism (C-compound and carbohydrate, nitrogen and lipid metabolism)</i>					
At3g11340	UGT76B1	52.9	UDP-glucuronosyl/UDP-glucosyl transferase family protein (Group D)	Nucleotide-sugar metabolism	Borner et al. (2003)
At1g66970		5.8	Glycerophosphoryl diester phosphodiesterase family protein	C-3 compound metabolism, phospholipid metabolism	
At5g24210	LPS	4.4	Lipase class 3 family protein	Lipid, fatty acid and isoprenoid metabolism	Ishiyama et al. (2004)
At5g05460		4.3	Glycosyl hydrolase family 85 protein	Chloroplast	
At2g30550		3.7	Lipase class 3 family protein	Lipid, fatty acid and isoprenoid metabolism; chloroplast	
At5g37600	ATGSRI	3.1	Encodes a cytosolic glutamine synthetase	Nitrate assimilation; metabolism of glutamine-glutamate	
<i>Transport</i>					
At4g13510	ATAMT1	3.9	Ammonium transport 1, encodes a plasma membrane localized ammonium transporter	Ammonium transport	Loque et al. (2006)

Table 2 continued

AGI	Alias	SA wt	Annotation	FunCat SubCategories	References
At5g53550	<i>YSL3</i>	3.7	Yellow stripe like 3; member of the oligopeptide transporter (OPT) family	Response to biotic stimulus; peptide transport	Waters et al. (2006)
At1g12940	<i>ATNRT2.5</i>	3.0	Nitrate transporter 2.5, member of High affinity nitrate transporter family	Nitrate transport	Pourtau et al. (2006)
<i>Unclassified</i>					
At1g14880		11.7	Similar to unknown protein	Unclassified protein	Rohde et al. (2004)
At3g29240		4.7	Similar to unknown protein	Unclassified protein	Vergnolle et al. (2005)
At3g62780		4.3	C2 domain-containing protein	Unclassified protein	
At4g32870		4.2	Similar to unknown protein	Unclassified protein	Yu et al. (2005)
At2g39210		4.0	Nodulin family protein	Unclassified protein	Taji et al. (2004)
At3g26600		3.7	Armadillo/beta-catenin repeat family protein	Unclassified protein	Coates (2003)
At1g49750		3.4	Leucine-rich repeat family protein	Unclassified protein	Wong et al. (2006)
At1g52780		3.3	Similar to unknown protein	Unclassified protein	Dunkley et al. (2004)
At3g13950		3.1	Similar to unknown protein	Unclassified protein	Sato et al. (2007)
At3g48640		3.0	Similar to unknown protein	Unclassified protein	

Only genes with induction ratio higher than 3 are included. Genes were classified in the more representative subcategories, as defined by MIPS (FunCat DB (Ruepp et al. 2004)) and ranked by decreasing degree of activation by SA

^a Gene previously reported by our group (Blanco et al. 2005) which induction ratio was quantified from Northern experiments; AGI, Arabidopsis Genome Initiative gene index; SA wt, average ratio SA/C in wt plants

Table 3 NPR1-independent SAIGs

AGI	Alias	SA wt	SA npr1	Annotation	FunCat Subcategories	References
<i>Oxygen and radical detoxification</i>						
At1g28480	<i>GRXC9</i>	11.3	3.4	Glutaredoxin family protein	Oxygen and radical detoxification; electron transport	Ndamukong et al. (2007)
At1g03850	<i>GRXS13</i>	10.5	3.2	Glutaredoxin family protein	Oxygen and radical detoxification; electron transport; chemoperception and response; cytokinin response; chloroplast	Brenner et al. (2005)
At2g47730 ^a	<i>AtGSTF8</i> (<i>GST6</i>)	3.2	3.3	Glutathione S-transferase (phi class)	Disease, virulence and defense; oxygen and radical detoxification; metabolism of toxins/drugs; protein modification; chloroplast	Uquillas et al. (2004); Thatcher et al. (2007)
At2g29420	<i>AtGSTU7</i> (<i>GST25</i>)	2.6	4.0	Glutathione S-transferase (tau class)	Metabolism of toxins/drugs; oxygen and radical detoxification; chemoperception and response; plant signaling molecules response	Wagner et al. (2002); Blanco et al. (2005)
<i>Cellular sensing and response to external stimulus/disease, virulence and defense</i>						
At1g31580	<i>ECSI</i>	9.1	3.4	Cell wall protein linked to a locus influencing resistance to Xcc750	Disease, virulence and defense; cell wall	Aufsatz et al. (1998)
At3g01420 ^a	<i>α-DOX1</i>	7.9	8.5	α -dioxygenase	Oxidative stress response; chemoperception and response; plant signaling molecules response; cell death; fatty acid metabolism	De Leon et al. (2002); Blanco et al. (2005)
At1g76680 ^a	<i>OPRI</i>	7.3	8.1	12-oxophytodienoate reductase 1	Chemoperception and response; plant signaling molecules response; fatty acid derivatives mediated signal transduction; senescence; lipid metabolism; metabolism of acetic acid derivatives	He and Gan (2001); Blanco et al. (2005)
At4g14400	<i>ACD6</i>	6.5	2.8	Accelerated cell death 6, a novel ankyrin protein	Chemoperception and response; response to biotic stimulus; plant signaling molecules response; protein binding; cell death	Lu et al. (2003)
At4g19660	<i>NPR4</i>	5.4	3.3	NPR1-like protein 4	Response to biotic stimulus; protein binding; nucleus	Liu et al. (2005); Zhang et al. (2006)
At1g77120	<i>ADH1</i>	2.3	2.7	Alcohol dehydrogenase 1	Osmotic and salt stress response; osmosensing and response; C-compound and carbohydrate metabolism; metabolism of vitamins, cofactors, and prosthetic groups; respiration	Liu et al. (2005)
At2g24850	<i>TAT3</i>	2.3	2.1	Tyrosine aminotransferase 3	Stress response; chemoperception and response; plant signaling molecules response; response to wounding; aromatic aminoacid metabolism; nitrogen metabolism	Titarenko et al. (1997)
At1g07400	<i>HSP17.8-CI</i>	2.1	2.2	17.8 kDa class I heat shock protein	Oxidative stress response; heat shock response; temperature perception and response	Stanley et al. (2005)

Table 3 continued

AGI	Alias	SA wt	SA nprl	Annotation	FunCat Subcategories	References
<i>Metabolism (Nucleotide-sugar metabolism, UGTs)</i>						
At1g05560 ^a	UGT75B1 (UGT1)	5.8	5.7	UDP-Glycosyl transferase (Group L) involved in callose synthesis at the cell plate	Chemoprotection and response; plant signaling molecules response; nucleotide-sugar metabolism; cell cycle; vesicle formation	Hong et al. (2001); Blanco et al. (2005)
At2g43820 ^a	UGT74F2	4.7	5.1	UDP-Glycosyl transferase (Group L); involved in the tryptophan synthesis pathway; glycosylates SA in vitro	Nucleotide-sugar metabolism	Quiel and Bender (2003); Blanco et al. (2005)
At4g15550	UGT75D1	4.0	3.0	UDP-Glycosyl transferase (Group L)	Nucleotide-sugar metabolism; chloroplast	Jackson et al. (2001)
At4g01070	UGT72B1	3.4	2.1	UDP-Glycosyl transferase (Group E)	Degradation/modification of foreign (exogenous) compounds; chemoprotection and response; nucleotide-sugar metabolism	Brazier-Hicks and Edwards (2005)
At4g34135 ^a	UGT73B2	2.9	3.7	Flavonol 7-O-glucosyltransferase (Group D)	Nucleotide-sugar metabolism	Blanco et al. (2005); Langlois-Meurinne et al. (2005)
At4g34138 ^a	UGT73B1	2.8	3.5	UDP-glycosyltransferase/abscisic acid glucosyltransferase (Group D)	Nucleotide-sugar metabolism	Blanco et al. (2005); Langlois-Meurinne et al. (2005)
<i>Metabolism (secondary, carbohydrate, aminoacid and others)</i>						
At5g24530	F3'H	13.1	3.5	Oxidoreductase, 2OG-Fe(II) oxygenase family protein, similar to flavanone 3-hydroxylase	Metabolism of stilbenes, flavonoids	Blanco et al. (2005); Langlois-Meurinne et al. (2005)
At5g51830		4.6	2.4	pfkB-type carbohydrate kinase family protein	C-compound and carbohydrate metabolism; acetate fermentation; phosphate metabolism	Bruneau et al. (2006)
At3g16150		3.4	2.2	L-asparaginase, putative	Aminoacid metabolism; nitrogen metabolism; protein/peptide degradation	
At3g14990		2.8	2.5	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein, thiamin biosynthesis	Biosynthesis of vitamins, cofactors, and prosthetic groups	
At4g21990	ATAPR3	2.3	3.6	Adenosine 5'-phosphosulfate reductase-like protein; it has a domain of glutathione (GSH)-dependent reductase	Sulfur metabolism; chloroplast	Houston et al. (2005)
<i>Transcription</i>						
At5g63790	ANAC102	5.3	4.7	Member of the plant-specific NAC-domain transcription factor family	Transcriptional control	Delessert et al. (2005)
At5g13080	ATWRKY75	3.3	2.3	Member of WRKY Transcription Factor; Group II-c	Transcriptional control; nucleus	Gechev et al. (2005); Devaiah et al. (2007)
<i>Unclassified</i>						
At2g25510		13.1	4.3	Unknown protein	Unclassified protein	Sato et al. (2007)
At4g13180	SDRLP	4.9	5.2	Short-chain dehydrogenase/reductase (SDR) family protein	Mitochondrion	Catarecha et al. (2007)
At5g64250	NPD	2.8	2.6	2-nitropropane dioxygenase family/NPD family	Unclassified protein	Shahollari et al. (2007)

Table 3 continued

AGI	Alias	SA wt	SA npr1	Annotation	FunCat Subcategories	References
At5g39050		2.5	2.2	Transferase family protein	Unclassified protein	Tamaaki et al. (2003)
At3g25190		2.1	2.6	Nodulin, putative	Unclassified protein	Ramsey et al. (2006)
At5g14730		2.0	2.8	Similar to unknown protein	Unclassified protein	Baerson et al. (2005)

All genes with induction ratio higher than 2 are included. Genes were classified in the more representative subcategories, as defined by MIPS (FunCat DB (Ruepp et al. 2004)) and ranked by decreasing degree of activation by SA

^a Genes previously reported by our group (Uquillas et al. 2004; Blanco et al. 2005) which induction ratio was quantified from Northern experiments; AGI, Arabidopsis Genome Initiative gene index; SA wt, average ratio SA/C in wt plants; SA npr1, average ratio SA/C in *npr1-1* mutant plants

than early NPR1-dependent SAIGs to a wide range of stress treatments (biotic, chemical and abiotic) that alter the redox cell homeostasis (Fig. 1). For example, early NPR1-independent SAIGs—in contrast to early NPR1-dependent SAIGs—were responsive to inoculation with *Botrytis cinerea* and to treatments with several chemical stressors (Fig. 1). In addition, this study showed that both groups of genes were responsive to SA but not to other hormones involved in stress (data not shown).

With the double purpose of corroborating the results of the microarray experiment regarding the responsiveness to SA and the requirement of NPR1 coactivator observed in, and analyzing the kinetics of this process, we selected a group of representative genes according to the level of transcript accumulation by SA and to their function. Among the chosen genes were 5 early NPR1-dependent SAIGs (*LLP*, *NIMIN1*, *NIMIN2*, *WRKY38*, *UGT76B1*) and 5 early NPR1-independent SAIGs (*GRXC9*, *GRXS13*, *F3'H*, *ANAC102*, *SDRLP*) with high levels of transcript accumulation and representative of different functional categories (Tables 2, 3). *OPR1* and *PR-1* were used as controls for NPR1 independence and dependence, respectively (Blanco et al. 2005). The experiment involved treatment of wild type and *npr1-1* mutant Arabidopsis seedlings with SA for different periods of time, and Northern blot analysis of transcripts levels (Fig. 2). As expected, all genes selected were activated by SA in wild type seedlings. We also confirmed that SA-induced activation of *WRKY38*, *UGT76B1*, *NIMIN1*, *NIMIN2*, *LLP* genes did not occur in the *npr1-1* mutant, while activation by SA of *SDRLP*, *ANAC102*, *GRXC9*, *GRXS13* and *F3'H* was detected in both genotypes (Fig. 2). Interestingly, the kinetic behavior of these genes after activation by SA was different and correlated with the degree of requirement of NPR1 for activation. Complete independence of NPR1 for SA-induced activation correlated with an earlier and transient activation by SA (*SDRLP*, *ANAC102* and *OPR1* in Fig. 2). As previously reported (Blanco et al. 2005), we found that activation of early NPR1-independent SAIGs by SA in the *npr1-1* mutant had a more sustained activation (Fig. 2), which correlated with higher accumulation of SA (data not shown). Genes with partial requirement of NPR1 showed an early but more sustained activation by SA (*GRXC9*, *GRXS13*, *F3'H* in Fig. 2). Consistent with our microarray data (Table 3), activation of these genes was detected in the *npr1-1* mutant but a functional NPR1 was required for maximum activation rate.

All genes classified as early NPR1-independent SAIGs had a peak of increase in mRNA levels around 1–2.5 h after SA treatment. In contrast, early NPR1-dependent SAIGs (*WRKY38*, *UGT76B1*, *NIMIN1*, *NIMIN2*, *LLP* in Fig. 2) displayed a more delayed kinetics (peak of mRNA levels after 5 h). The activation kinetics of both classes of

Table 4 Functional categories over-represented in the groups of early NPR1-dependent and early NPR1-independent SA-induced genes, compared to the complete Arabidopsis genome

Term	Observed frequency	Expected frequency	P-value
NPR1-Dependent-SAIGs			
<i>Metabolism</i>	35.2%	17.3%	1.61e−07
Phosphate metabolism	24.4%	6.55%	1.41e−07
<i>Protein fate</i>	28.0%	10.9%	3.31e−05
Protein modification	26.0%	7.61%	5.21e−05
Modification by phosphorylation, dephosphorylation, autophosphorylation	19.8%	4.37%	2.23e−05
<i>Cell rescue, defense and virulence</i>	12.2%	5.01%	5.17e−03
Disease, virulence and defense	8.67%	1.61%	3.19e−06
<i>Interaction with the environment</i>	13.2%	5.81%	7.24e−03
Cellular sensing and response to external stimulus	13.2%	5.24%	1.36e−03
Chemoperception and response	8.67%	2.95%	8.73e−03
Response to biotic stimulus	6.12%	1.04%	1.48e−04
<i>Systemic interaction with the environment</i>	7.65%	2.66%	2.81e−02
Plant/fungal specific systemic sensing and response	7.14%	2.43%	3.69e−02
<i>Cell fate</i>			
Cell death	3.06%	0.35%	9.95e−03
Total genes	197	28413	
NPR1-Independent-SAIGs			
<i>Metabolism</i>	54.8%	17.3%	1.77e−05
C-compound and carbohydrate metabolism	25.8%	5.79%	2.08e−02
<i>Cell rescue, defense and virulence</i>	32.2%	5.01%	1.22e−04
Detoxification	12.9%	0.93%	1.52e−02
Oxygen and radical detoxification	12.9%	0.91%	1.42e−02
<i>Interaction with the environment</i>	35.4%	5.81%	5.28e−09
Cellular sensing and response to external stimulus	35.4%	5.24%	1.91e−09
Chemoperception and response	29.0%	2.95%	1.45e−09
<i>Systemic interaction with the environment</i>	22.5%	2.66%	1.06e−03
Plant/fungal specific systemic sensing and response	22.5%	2.43%	5.99e−04
Plant hormonal regulation	22.5%	2.07%	2.10e−04
Total genes	31	28413	

The over-represented functional categories were obtained according to the MIPS classification using BioMaps tool from the VirtualPlant webpage (<http://virtualplant.bio.nyu.edu>). Observed frequency refers to the list of SAIGs analyzed and expected frequency refers to the whole Arabidopsis genome. Hypergeometric method and a Bonferroni correction were used for the analysis with a cutoff of 0.05

early SAIGs was clearly distinguishable from that of *PR-1* gene, a marker for “late NPR1-dependent SAIGs” (Fig. 2).

SA-mediated activation of selected SAIGs

To study the mechanism of SA-induced activation we selected six SAIGs: two completely independent of NPR1 (*ANAC102* and *SDRLP*), two genes partially dependent on this coactivator (*GRXC9* and *GRXS13*) and two completely dependent on NPR1 for activation (*NIMIN1* and *LLP*).

In the first place, we determined if activation of these genes required de novo protein synthesis. Wild-type seedlings were pretreated for 1 h with the protein synthesis inhibitor cycloheximide (CHX) (or MS as a control), and then treated for 2.5, 8 or 24 h with SA (or MS as a control). After this, the steady state levels of transcripts were

determined by Northern blot. Activation of *SDRLP*, *ANAC102*, *GRXS13*, *GRXC9* and *NIMIN1* genes by SA was not inhibited by CHX (Fig. 3a). Therefore, SA increases the mRNA levels of these genes by a mechanism independent of de novo protein synthesis and likely controlled by modification of pre-existing factors. Interestingly, the genes *ANAC102* and *GRXC9* were strongly activated by CHX in the absence of SA (Fig. 3a). This behavior was previously reported by us and others in early SAIGs of Arabidopsis and tobacco (Qin et al. 1994; Horvath et al. 1998; Uquillas et al. 2004). As judged by information from public microarray database, it seems to be a common response of a large number of early SAIGs (see response to CHX in Fig. 1). A possible explanation for this effect is that one of the mechanisms that controls transcription of these early SAIGs is basal repression by a

Fig. 1 Expression profiles of SAIGs obtained from public microarray database. The expression profiles of the NPR1-dependent SAIGs whose induction was >3 -fold (see Table 2) and of all NPR1-independent SAIGs identified in this study (see Table 3) were analyzed on microarray experiments selected from the GeneVestigator database (Laule et al. 2006). Experiments with other stress treatments, either hormonal (ABA, ACC, ethylene, GA3, IAA), biotic

or abiotic, in which SAIGs showed no response were not included. Expression levels ($\log_2$ ratio) are indicated by a color code shown at the bottom of the figure. The number of arrays used in each experiment is indicated at the right side. Genes with highest induction level in each group are marked with a green box (AT1G28480 for NPR1-independent and AT5G03350 for NPR1-dependent group)

labile protein, whose effect can be relieved by SA (see model in Fig. 6). In the case of *LLP*, pretreatment with CHX partially inhibited the activation by SA. This is compatible with a mixed activation mechanism. In contrast, we found that SA-mediated activation of *PR-1*, a marker for late genes, was completely inhibited by CHX, as previously reported (Qin et al. 1994).

To determine if the early increases of mRNA levels of selected SAIGs caused by SA result from mRNA stabilization or from transcriptional activation, we analyzed the expression of these genes in the presence of cordycepin, a transcriptional inhibitor (Gutierrez et al. 2002). Wild type

seedlings were treated with SA for 2.5 h to produce the maximum transcript accumulation, and then incubated for different periods of time with SA or MS medium (control) in the presence or absence of cordycepin (0.6 mM). The mRNAs were detected by Northern blot analysis and quantified by a Phosphorimager. The effect of cordycepin was controlled by analyzing the levels of *NIA2* mRNA (nitrate reductase 2), a constitutively expressed gene not responsive to SA. In the absence of cordycepin, removal of SA produced a decay of mRNA levels for all SA-responsive genes (Fig. 3b, compare C vs. SA in left panels). When transcription was inhibited by cordycepin, a similar

Fig. 2 Time-course of SA-induced expression of selected SAIGs in wild type and *npr1-1* plants. RNA was isolated from 15 day-old plants either treated with 0.5 mM SA (SA) or maintained in MS medium (C) for the times (hours) indicated at the top of the figure. Ten micrograms of total RNA were loaded per lane, and the blots were hybridized with specific probes for the following genes: *SDRLP* (AT4G13180), *ANAC102* (AT5G63790), *GRXC9* (AT1G28480), *GRXS13* (AT1G03850), *F3'H* (AT5G24530), *WRKY38* (AT5G22570), *UGT76B1* (AT3G11340), *NIMIN-1* (AT1G02450), *NIMIN-2* (AT3G25882) and *LLP* (AT5G03350). *OPR1* (AT1G76680) and *PR1* (AT2G14610) were used as controls for NPR1 independence and dependence, respectively. *ACTIN 3* (AT3G53750) was used as a loading control

decay of mRNA levels was detected in the absence or in the presence of SA for all genes analyzed (Fig. 3b, compare C vs. SA in right panels). To support this conclusion, we also calculated mRNA half-lives ($t_{1/2}$) for all genes analyzed, in the absence and in the presence of SA, using three independent biological samples. For this, we quantified mRNA levels by Phosphorimager and the $t_{1/2}$ values were calculated according to Lidder et al. (2005). Results from this analysis indicate that SA does not affect $t_{1/2}$ of SAIGs mRNAs (data not shown). These results are consistent with the idea that SA does not increase mRNA levels of early SAIGs through increasing mRNA stability, and support the idea that SA induces gene activation by exerting a transcriptional control.

We then determined whether the subclass II of TGA transcription factors (TGA2, TGA5 and TGA6) was involved in the transcriptional activation or de-repression of these early genes by SA. For this purpose, we compared the response of selected genes to SA treatment in wild type and *tga2-1/tga5-1/tga6-1* triple mutant Arabidopsis seedlings (Zhang et al. 2003). As shown in Fig. 4, activation of the early NPR1-independent (*SDRLP*, *ANAC102*, *GRXC9* and *GRXS13*) and the early NPR1-dependent (*LLP* and

Fig. 3 Analysis of SA-induction mechanism for 6 selected SAIGs. **a** Effect of the inhibitor of protein synthesis, cycloheximide (CHX) on SAIGs expression. 15 day-old wild type seedlings were pretreated for 1 h with CHX (six lines on the right side) or MS (control, six lines on the left sides) and then incubated for 2.5, 8 or 24 h with 0.5 mM SA or MS as a control (C), either in the presence or absence of CHX. Ten μ g of total RNA were loaded per lane, and the blots were hybridized with specific probes for the genes indicated in the figure. *PR1* and 18S rRNA genes were used as controls for CHX treatment and loading, respectively. **b** Effect of the transcriptional inhibitor cordycepin on SAIGs expression. 15 days-old wild type seedlings were pretreated with 0.5 mM SA or MS as a control (C) for 2 h. Then, seedlings were transferred to MS or 0.5 mM SA in the absence (left panel) or presence of 0.6 mM cordycepin (right panel) for the times (hours) indicated at the top of the figure. RNA was analyzed as described above. *NIA2* (AT1G37130) and *ACTIN 3* (AT3G53750) were used as controls of cordycepin effect and loading, respectively

NIMIN1) SAIGs was severely impaired in the *tga2-1/tga5-1/tga6-1* triple mutant, indicating involvement of TGA2/5/6 in the transcriptional activation of these genes. In contrast, an increase in basal levels and impairment of SA-activation at 8, but not at 24 h post treatment, was detected for *PR-1* gene in the *tga2-1/tga5-1/tga6-1* triple mutant (Fig. 4). This behavior is consistent with the possibility that the TGA2/5/6 factors are involved in both the basal repression and the SA-mediated activation of *PR-1*

Fig. 4 SA-induced expression of selected SAIGs in *tga6-1/tga2-1/tga5-1* plants. RNA was isolated from 15 day-old plants either treated with 0.5 mM SA (SA) or maintained in MS medium (c) for the times (hours) indicated at the top of the figure. Ten micrograms of total RNA were loaded per lane, and the blots were hybridized with specific probes for the indicated genes. *ACTIN 3* was used as a loading control

(Zhang et al. 2003; Rochon et al. 2006), but it also suggests the involvement of other factors in late activation of *PR-1* by SA (Wang et al. 2005).

SA-induced genes revealed common *cis*-elements in their promoters

Having found that the SAIGs selected in this study were transcriptionally activated by SA through a TGA-dependent mechanism, we looked for common putative *cis*-elements in the promoter sequences of both groups of SAIGs. We searched for over-represented motifs (16 and 20 bp length) separately in the 31 early NPR1-independent SAIGs (Table 3) and in the 197 early NPR1-dependent SAIGs (supplementary Table S1), with the software MotifSampler (Thijs et al. 2002). Over-represented motifs were ranked according to their log-likelihood score, which considers the conservation of the motifs and the number of copies of each motif found in the input sequences. Table 5 shows the 10 best-ranked motifs for each group of genes. To determine if the motifs found in this study correspond to defined regulatory elements already described in plant promoters, we searched for these motifs in PLACE and TRANSFAC databases (Wingender et al. 1996; Higo et al. 1999). Interestingly, the 10 best ranked 20 bp motifs found in the early

NPR1-independent SAIGs, matched the *as-1*-like element described in the 35S promoter of the cauliflower mosaic virus (Qin et al. 1994). This is in agreement with results we described before for a smaller group of early NPR1-independent SAIGs (Blanco et al. 2005). On the other hand, for early NPR1-dependent SAIGs, the 10 best ranked 16 bp motifs found matched the core of TL-1 element (Table 5). This element was recently described as a consensus sequence over-represented in the promoter of a group of 13 NPR1-responsive ER-resident genes (Wang et al. 2005).

Early SAIGs are activated in the defense reaction triggered by an avirulent bacterium

Arabidopsis plants activate SA biosynthesis as part of their defense reaction to the attack of avirulent pathogens (Summermatter et al. 1995). We wanted to know whether the expression of the early SAIGs identified here increased during the activation of pathogen-induced defenses. For this purpose, we inoculated Arabidopsis plants with the avirulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 that carries the *AvrRpm1* gene (*Pst* *AvrRpm1*, 5×10^6 cfu/ml); control plants were mock inoculated. RNA samples were obtained at 0, 5, 21 and 48 h post inoculation (hpi) and transcript levels of the early SAIGs were detected by Northern blot. As shown in Fig. 5a, activation of all SAIGs analyzed (*SDRLP*, *ANAC102*, *GRXS13*, *GRXC9*, *NIMIN1* and *LLP*) was detected starting 5 h post infection (hpi). SA levels were measured by HPLC in pathogen-treated samples collected at 5, 26 and 48 hpi. Increase in SA levels was detected from 5 hpi (Fig. 5b). All genes, except *GRXS13*, showed a peak of activation earlier than *PR-1*. These results support a role for SAIGs in the defense response to pathogen infection.

Discussion

It has been clearly established that the rise of SA levels and the subsequent induction of genes triggered by this hormone (SAIGs) are essential steps for the development of a defense reaction to pathogen infection (SAR), and to other stressful environmental conditions (Durrant and Dong 2004; Loake and Grant 2007). Although important knowledge concerning SA effects has been obtained in the last years, essential elements of its action in the cellular response to stress are still unknown (Loake and Grant 2007). Looking for clues to understand SA role in defense to stress, we have focused our attention in the early responses triggered by this hormone. We previously described two pathways for rapid activation of SAIGs; one dependent on the NPR1 coactivator and other independent of it (Uquillas et al. 2004; Blanco et al. 2005; Holuigue et al. 2007).

Table 5 In silico promoter analysis of early SAIGs

Motif Sampler analysis ^a	# Promoters	Instances
NPR1-independent genes		
nwn ACGT nnnyrnk ACGT	20	41
n CGT nnnyrn GACGT nnnn	17	38
ynm TnACGT nnnyrn ACG	17	34
CGT mwncnn GACG nnnnmn	15	32
wn GTC rnnyrmwn ACn wCnwn	14	29
nnnwn GTC nnnyrn TnACG w C	14	34
CnT nnnyrmnn ACGTCA kn A	13	31
nnn GTC rnnyrn AnG wCnn	13	28
nnn GwC rTnnnyr AyGAC nn	13	28
mnn ATG wnGnm AnCrnTGAC	12	22
---CGT---C---GAC--- (consensus sequence)		
TGACGTCA-C--TGACGTCA (perfect <i>as-1</i> palindrome)		
NPR1-dependent genes		
snyTny TyTnyTTynTynT	83	108
nnTynnn TCTTC nTyny T	78	99
TCTnyTTynTynTynTyyTy	74	100
nyyny TTCTTC nnnyTy Tny	72	90
nTyTny TTYTTYTTYTnyTT	72	98
TyTnTynTCTyyTynyn	72	86
nnnyy TCTnyyTyyTyyTCT	72	93
nCnny TTCTnyTTCyTnnny	70	90
nTy TTYyTyTTYTynTyT	71	92
yyy TnTnTyTyynTCTnyy	72	93
TTCTTCTTCAG (TL1 element)		

^a Selection of the 10 highest scored 20 bp-motifs over-represented in the promoters of the 31 NPR1-independent and the 197 NPR1-dependent genes identified. R = A or G; Y = C or T; S = G or C; W = A or T; K = G or T; M = A or C; B = C or G or T; D = A or G or T; H = A or C or T; V = A or C or G; N = any base.

The transcriptomic and expression analyses described in this article further support the existence of these two pathways for rapid activation of SAIGs; one independent of NPR1 used by 14% of these genes, and the other dependent of NPR1 used by the rest of them. Here we report that these two groups of genes, not only differ in their main functional categories, but also in their timing and mechanism of activation by SA. Considering this and previous published work (Niggeweg et al. 2000; Pontier et al. 2001; Uquillas et al. 2004; Blanco et al. 2005; Thurow et al. 2005; Wang et al. 2005, 2006; Butterbrodt et al. 2006; Rochon et al. 2006) we propose a model to explain the differences in the SA-mediated activation of these two classes of early SAIGs (Fig. 6).

Role of early SAIGs in the stress defense response

Several functions in the defense response to stress have been attributed to the different classes of SAIGs. An antimicrobial role has been reported for *PR* genes, the well

known group of genes belonging to the class of late NPR1-dependent SAIGs (Maleck et al. 2000; van Loon et al. 2006). SAIGs recently identified as primary targets of NPR1, which can be classified as early NPR1-dependent SAIGs, code for proteins from the protein secretory machinery and WRKY factors (Wang et al. 2005, 2006). The identification of early SAIGs (NPR1-dependent and NPR1-independent pathways) described in this work supports the idea that SA plays additional roles in defense and acclimatory responses to stress, such as recovery of the cell redox balance, intracellular stress signaling, improvement of pathogen recognition, and promotion of metabolic changes. Based on the expression analysis of a selection of these genes in response to an avirulent bacterial pathogen, we propose that early SAIGs may contribute to generate a pathogen-induced defense reaction. Intersection of our list of 228 early SAIGs with the list of 1,187 genes activated after 8 h of treatment with BTH (Wang et al. 2006), indicates that 88 out of the 228 genes (38%) had not been

Fig. 5 Expression of SAIGs and SA levels in Arabidopsis plants inoculated with *Pseudomonas*. Six week-old plants were inoculated with *Pseudomonas syringae* pv tomato DC3000 carrying the AvrRpm1 gene (Pst AvrRpm1, 5×10^6 cfu/ml) or with $MgCl_2$ (mock). Then, leaf samples were collected at the indicated times for RNA extraction and Northern blot analysis (a), and for measurement of SA levels by HPLC (b). SAG, glycosylated-SA. Averages and standard deviations of SA and SAG levels were calculated from three independent biological replicates

previously considered as BTH-dependent SAIGs. A considerable proportion of the genes identified here (133 genes, 58%) were also detected as ozone-responsive genes (Mahalingam et al. 2005), indicating that early SAIGs also play a role in the response to ozone treatment. We will focus our discussion in some of the functions early activated by SA, which could be more relevant to the role of this hormone in defense and acclimatory responses.

The regulation of changes in the cellular redox status is one of the roles recently attributed to SA in pathogen-induced defenses and light acclimatory responses in Arabidopsis (Durrant and Dong 2004; Fobert and Despres 2005; Foyer and Noctor 2005; Mateo et al. 2006; Holuigue et al. 2007). This regulatory role seems relevant to SA function, considering that this hormone is also produced and required for defense responses to different stressful conditions

associated to cellular oxidative stress, such as UV irradiation, ozone exposure and high light (Yalpani et al. 1994; Surplus et al. 1998; Rao and Davis 1999; Mateo et al. 2006; Janda et al. 2007). A pro-oxidative activity of SA recognized more than 15 years ago (Chen et al. 1993) gives support to the positive feedback interaction between SA and reactive oxygen species (ROS) associated to cell death (Shirasu et al. 1997; Overmyer et al. 2003; Dat et al. 2007). Nevertheless, evidences of an antioxidant activity of SA associated to defense and acclimation processes have appeared only in the last years (Despres et al. 2003; Mou et al. 2003; Yang et al. 2004; Mateo et al. 2006; Tada et al. 2008). The molecular basis for this antioxidant activity is still unknown.

Results reported here support an antioxidant role for SA and strengthen previous evidences showing that SA triggers rapid activation of genes with antioxidant and detoxifying activities, through an NPR1-independent pathway (Uquillas et al. 2004; Blanco et al. 2005; Holuigue et al. 2007). Interestingly, in this class of early NPR1-independent SAIGs we detected genes coding for two glutaredoxins (*GRXC9* and *GRXS13*), two glutathione *S*-transferases (*AtGSTU7* and *AtGSTF8*) and 6 UDP-glycosyl transferase (*UGT72B1*, *UGT75B1*, *UGT75D1*, *UGT74F2*, *UGT73B2*, *UGT73B1*) (Table 3).

GRXs, together with thioredoxins (TRX), are the two major enzymatic systems that catalyze reversible thiol-based reduction of target proteins (Rouhier et al. 2008). These proteins have been allegedly implicated in the SA-dependent reduction of NPR1 and TGA factors required for the transcription of defense genes (Fobert and Despres 2005; Foyer and Noctor 2005; Tada et al. 2008). The glutaredoxin *GRXC9* (coded by At1g28480, also named GRX480) was recently reported to be induced by SA and to interact with TGA factors (Ndamukong et al. 2007). Ectopic expression of *GRXC9* suppresses JA-responsive PDF1.2 transcription, suggesting that this enzyme could be a key piece in the SA/JA cross-talk (Ndamukong et al. 2007). Here we report that *GRXC9* gene is rapidly activated by SA (peak 1–2.5 h, see Fig. 2), and by infection with an avirulent pathogen (Fig. 5). We also found that activation of *GRXC9* by SA is partially dependent on NPR1 function, because considerable levels of *GRXC9* mRNA were detected in the *npr1.1* mutant compared to the wild type (50% at 2.5 h, Fig. 2). In contrast, Ndamukong et al. reported that activation of *GRXC9* by SA is almost negligent in the *npr1.1* background (Ndamukong et al. 2007). These differences could be due to differences in the experimental conditions used in both experiments; we used 15 days-old seedlings treated with 0.5 mM SA, while Ndamukong et al. used 3 week-old plants treated with 1 mM SA.

The other glutaredoxin gene (*GRXS13*, At1g03850), which is also included in the group of early NPR1-independent SAIGs, codes for an enzyme whose function in defense has not been studied. Here we show that SA

Fig. 6 Mechanistic model for SA-mediated early activation of genes. **a** Temporal course of the reaction after SA addition is indicated by the color bar at the top of the figure. Windows corresponding to peaks of induction of two classes of early SAIGs are indicated. “Early NPR1-independent SAIGs” are rapidly and transiently activated by SA by reversible recruitment of a coactivator (CoA) to the TGA2/*as-1*-like element (T2/*as-1*) complex. Putative inhibitors (I) that recognize CoA and/or TGA2 could be involved. **b** “Early NPR1-dependent SAIGs”

are activated by SA via recruitment of activated NPR1 (NPR1_A) to form the transcriptionally active NPR1_A-T2-TGA ternary complex. We also propose involvement of a putative transcriptional activator (TL1-A) that recognizes the TL-1 element. *as-1*-like element: two TGACGTCA-like motifs spaced by 4 nucleotides; TGA box: TGACG; TL-1 box: TTCTTCTTC. Red arrows represent activation and blocked red arrows represent repression

activates *GRXS13* with an early and sustained kinetics (Fig. 2). Similarly, a strong and sustained activation of this gene was also detected after pathogen infection (Fig. 5). As with *GRXC9*, we included *GRXS13* in the list of early NPR1-independent SAIGs, due to the considerable levels of activation by SA detected in the *npr1-1* mutant (Fig. 2).

We found that SA also produces a rapid activation of two GSTs and six UGTs, by an NPR1-independent pathway. These transferases catalyze the formation of S-glutathionylated and O-glycosylated conjugates of different acceptor molecules. As we previously discussed (Blanco et al. 2005; Holuigue et al. 2007), these enzymes have been implicated in the detoxification of endogenous and xenobiotic compounds, being good candidates to diminish toxic effects of metabolites (including ROS) in the stress defense reaction (Lim and Bowles 2004; Edwards et al. 2005).

In sum, a rapid induction of this group of 10 enzymes (GRXs, GSTs and UGTs) following the rise in SA levels during the pathogen-induced defense reaction could contribute to recover the cellular redox balance and to generate the reducing environment for NPR1/TGA reduction required for the activation of late SAIGs responsible for stress resistance (Despres et al. 2003; Mou et al. 2003).

This study indicates additional functions of the rapid cellular response triggered by SA. A role for SA in improving pathogen recognition and signaling for defense response is also suggested by the identification of genes coding for 11 disease resistance (R) proteins, 36 protein

kinases and 28 proteins with protein binding functions among the early SAIGs. Activation of genes coding for transcription factors belonging to different families (8 WRKY, 4 NAM, 3 ERF/AP2 and 2 myb) is also part of the rapid response triggered by SA. Only two of these genes are activated by an NPR1-independent pathway, *ANAC102* (NAM family) and *WRKY 75*. Here we show that *ANAC102* is rapidly activated by SA (Fig. 2) and also by treatment with an avirulent pathogen (Fig. 5). Although *ANAC102* has not been previously characterized, is highly homologous to *ATAF2*, previously described as a repressor of *PR* genes in *Arabidopsis* (Delessert et al. 2005). *WRKY75*, together with the NPR1-dependent *WRKY 46*, are the only WRKYs detected here as early SAIGs, that have not been previously associated to pathogen-induced defense responses (Eulgem and Somssich 2007). *WRKY75* has been reported to be involved in H₂O₂-induced cell death and as a modulator of phosphate acquisition and root development (Gechev et al. 2005; Devaiah et al. 2007). The function of *ANAC102* and *WRKY75* in the defense reaction in *Arabidopsis* remains to be characterized.

Mechanisms for defense genes activation triggered by SA

The results of the genome-wide analysis reported here give further support to the existence of different mechanisms responsible for the rapid activation of SAIGs. Based on the

study of representative members of the SAIGs identified in this work, we propose that SA-mediated gene activation involves transcriptional activation and/or de-repression. The mechanisms involved differ in their requirement for NPR1, TGA factors and SA-responsive promoter elements. Based on this and on previous mechanistic studies, we propose an explanatory model for the SA-induced activation of the two classes of early SAIGs that takes place at different times after exposure to SA (Fig. 6).

A cluster of 31 genes corresponding to the class of early NPR1-independent SAIGs, activated by SA after 0.5–2.5 h of treatment, has been identified in this (Table 3) and previous work from our group (Uquillas et al. 2004; Blanco et al. 2005). We propose that early transcriptional activation of these genes by SA is controlled by the *as-1*-like element (TGACGTCAnnnnTGACGTCA) that is over-represented in the group of 31 promoters (Table 5). As we previously discussed (Blanco et al. 2005), *as-1* is structurally and functionally different from isolated TGA boxes (TGACG) found in *PR-1* promoter (Krawczyk et al. 2002; Butterbrodt et al. 2006). *as-1* element confers immediate early responsiveness to SA (Qin et al. 1994) by a mechanism involving oxidative signals (Garreton et al. 2002). We think that binding of a member of the subclass II of TGA factors (TGA2/5/6) to the *as-1*-like element is required for activation of these genes by SA. This is supported by our evidence that activation of *SDRLP*, *ANAC102*, *GRXC9*, and *GRXS13* (representative early NPR1-independent SAIGs) in Arabidopsis is severely impaired in the *tga2-1/tga5-1/tga6-1* triple mutant (Fig. 4). Similarly, evidence obtained in tobacco indicates that TGA2.2, member of the subclass II of TGA factors in this species, is the main component of the *as-1* binding activity in nuclear extracts (Niggeweg et al. 2000) and is essential for activation of a couple of tobacco early genes (Pontier et al. 2001; Thurrow et al. 2005). The question that remains open is how SA activates transcription of early NPR1-independent SAIGs mediated by TGA2/5/6-*as-1* interaction. One possibility is that SA activates binding of TGA2/5/6 to the *as-1* element. This possibility has been explored in tobacco with contradictory results (Jupin and Chua 1996; Stange et al. 1997; Garreton et al. 2002; Butterbrodt et al. 2006). Furthermore, it must be mentioned that neither TGA2 from Arabidopsis (Rochon et al. 2006) nor TGA2.2 from tobacco (Thurrow et al. 2005) have transactivation activity. Therefore, other protein(s) must provide the transactivation domain to the TGA2/5/6-*as-1* complex. In sum, as shown in Fig. 6, we propose that SA activates early NPR1-independent SAIGs by recruitment of a co-activator protein (CoA) to the TGA2-*as-1* complex already formed under basal conditions. The presence of an inhibitor, which sequesters the coactivator or blocks the TGA2-*as-1* complex under basal conditions has been previously proposed in tobacco

(Johnson et al. 2001; Butterbrodt et al. 2006) and is consistent with the activation induced by CHX found in some of the early genes (Fig. 1 and (Uquillas et al. 2004).

Genes belonging to the class of early NPR1-dependent SAIGs have been identified in this (Table 2 and supplementary Table S1) and in previous studies oriented to identifying primary targets of NPR1 (Wang et al. 2005, 2006). Representative members of this group show a peak of induction between 5 and 12 h post SA treatment (*WRKY38*, *UGT76B1*, *NIMIN1*, *NIMIN2*, *LLP* in Fig. 2; *BiP2* in (Wang et al. 2005)), while induction of *PR-1* gene, a marker for late NPR1-dependent SAIGs, peaks after 24 h of treatment (Fig. 2).

A new SA-responsive promoter element named TL-1 element (TTCTTCTTC) was found to be over-represented in a group of early NPR1-dependent SAIGs (Wang et al. 2005). This element is functional in the Arabidopsis *BiP2* promoter where SA activates binding of factors to TL-1 element by promoting its translocation to the nucleus (Wang et al. 2005). The nature of the factor(s) that recognize this TL-1 sequence is still unknown. Interestingly, we found the same TL-1 element over-represented in the group of 197 early NPR1-dependent SAIGs identified in this work (Table 5). Furthermore, expression analysis of *NIMIN1* and *LLP* (representative early NPR1-dependent SAIGs) in response to SA in the *tga2-1/tga5-1/tga6-1* triple mutant also indicates involvement of TGA2/5/6 factors in the activation of these genes by SA (Fig. 4). We propose that TGA boxes (TGACG), such as those found in the Arabidopsis *PR-1* promoter, are recognized by TGA2/5/6 factors in promoters of early NPR1-dependent SAIGs (Fig. 6). In this case, requirement of NPR1 for activation of these genes by SA (Fig. 2), together with data from the literature indicating that NPR1 provides the transactivation activity to the TGA2-TGA box complex (Rochon et al. 2006), allow us to propose involvement of the NPR1-TGA2-TGA box ternary complex in the activation by SA of early NPR1-dependent SAIGs (Fig. 6). In this case, the question of how is NPR1 activated/reduced without de novo protein synthesis at the time these genes are induced remains open.

Mechanistic studies performed with *PR1* gene, a marker for the group of late NPR1-dependent SAIGs (Lebel et al. 1998; Maleck et al. 2000; Rochon et al. 2006; Wang et al. 2006; Eulgem and Somssich 2007; Kesarwani et al. 2007) support the involvement of positive and negative W and TGA boxes in its SA-mediated activation, recognized by WRKY and TGA2/5/6 factors.

Taken together, our findings support the idea that SA can use different mechanisms to activate early and late SAIGs. Proper combination of SA-responsive elements (*as-1*-like, TGA box, W box and TL-1 element) in SAIGs promoters which are recognized by factors that act as repressors, or by complexes of factors and co-activators acting as enhancers,

seems to be responsible for SA-controlled expression of these genes. Temporal control of gene expression by SA could be explained by reversible changes in the cellular redox homeostasis produced by SA. Interestingly, components of the transcriptional machinery involved in the expression of early and late SAIGs, such as NPR1, TGA factors, TGA box and *as-1*-like elements, seem to act as redox sensors for temporal control of gene expression by SA.

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