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Identification of NPR1-dependent and independent genes early induced by salicylic acid treatment in Arabidopsis

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Abstract

Salicylic acid (SA) plays a crucial role in stress resistance in plants by modifying the expression of a battery of genes. In this paper, we report the identification of a group of early SA-regulated genes of *Arabidopsis* (activated between 0.5–2.5 h), using the cDNA-*amplified fragment length polymorphism* technique (cDNA-AFLP). Using 128 different primer combinations, we identified several genes based on their differential expression during SA treatment. Among these, we identified 12 genes up-regulated by SA whose patterns of induction were confirmed by Northern analysis. The identified genes can be grouped into two functional groups: Group 1: genes involved in cell protection (i.e. glycosyltransferases, glutathion *S*-transferases), and Group 2: genes involved in signal transduction (protein kinases and transcription factors). We also evaluated NPR1 requirement for the induction of the 12 up-regulated genes, and found that only those belonging to Group 2 require this co-activator for their expression. *In silico* analysis of the promoter sequences of the up-regulated genes, allowed us to identify putative *cis*-elements over-represented in these genes. Interestingly, *as-1*-like elements, previously characterized as SA-responsive elements, were specifically over-represented in Group 1 genes. The identification of early SA-regulated genes is an important step towards understanding the complex role of this hormone in plant stress resistance.

Abbreviations: AFLP-TP, amplified fragment length polymorphism-based transcript profiling; *as-1*, activation sequence-1; GST, glutathione *S*-transferase; GT, glycosyltransferase; HR, hypersensitive response; *IEGT*, immediate early glycosyltransferase gene; JA, jasmonic acid; NPR1, nonexpressor of PR genes 1; PR, pathogenesis-related proteins; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; TDF, transcript derived fragments; TGAs, family of bZip transcription factors recognizing TGACG motif; WT, wild type

Introduction

Salicylic acid (SA) accumulation in plants is essential to establish effective defense responses against several kinds of environmental stresses such as pathogen attacks, UV irradiation, osmotic stress or ozone exposure (Gaffney *et al.*, 1993; Yalpani *et al.*, 1994; Borsani *et al.*, 2001). The relevance of SA in these defense responses has been clearly demonstrated by studying the effect of

direct SA treatment in wild type (WT) plants or through the analysis of mutant and transgenic plants where the accumulation of SA, or the function of SA signal transduction factors, have been impaired (reviewed in (Alvarez *et al*., 1998; Overmyer *et al*., 2003; Durrant and Dong, 2004).

Most of our current knowledge regarding the role of SA in defense responses has been obtained from the study of plant responses to pathogen attacks. After pathogen recognition, a local resistance reaction known as hypersensitive response (HR) is triggered. HR is preceded by local accumulation of SA and reactive oxygen species (ROS), and is characterized by rapid cell death around the site of infection to stop pathogen spreading. Subsequently, plants can develop resistance to secondary infections in distal tissues. This secondary resistance is termed systemic acquired resistance (SAR), is strongly dependent on SA accumulation, and is also characterized by a secondary oxidative burst (Alvarez *et al*., 1998; Durrant and Dong, 2004). SA and ROS have been proposed to be interconnected through a positive feedback loop that amplifies and regulates the intensity of HR, SAR and plant responses to other stresses (Draper, 1997; Overmyer *et al*., 2003).

Sialicylic acid triggers the induction of a number of genes (Maleck *et al*., 2000). Among these genes, the best characterized families encode proteins with antimicrobial activity known as pathogenesis-related proteins (PRs) (Van Loon and Van Strien, 1999), and detoxifying or antioxidant enzymes such as glutathione *S*-transferases (GSTs) and glycosyltransferases (GTs) (Edwards *et al*., 2000; Li *et al*., 2001). All these genes are not only induced by SA but also by pathogens (Maleck *et al*., 2000). *GSTs* and *GTs* are additionally induced by auxin, H_2O_2, different xenobiotics, and wounding (Horvath and Chua, 1996; Xiang *et al*., 1996; Delessert *et al*., 2004; Uquillas *et al*., 2004).

Studies carried out in tobacco related to the induction of *PRs*, *GSTs* and *GTs* by SA, allowed the grouping of these genes into two different classes according to their kinetics of induction: immediate early and late genes. Late genes, like *PR-1*, are induced after 4–6 h of SA treatment and their activation requires *de novo* protein synthesis (Uknes *et al*., 1993; Qin *et al*., 1994). Immediate early genes such as *GST1*(*GNT35*) and *IEGT* are activated after 30 min of SA treatment, and do not require *de novo* protein synthesis (Xiang *et al*., 1996; Horvath *et al*., 1998).

Despite the difference in activation kinetics, related SA-responsive elements containing TGACG-like motifs have been found to be functional in the promoter of both immediate early and late genes (Droog *et al*., 1995; Lebel *et al*., 1998; Strompen *et al*., 1998; Chen and Singh, 1999). The first of these motifs, named *as-1*, was described in the CaMV 35S promoter and contains two palindromic TGACGTCA tandem motifs (Lam *et al*., 1989). *as-1*-like motifs are recognized by bZIP transcription factors belonging to the TGA family. The *in vivo* relevance of *as-1*/TGAs interaction was demonstrated using dominant negative forms of TGA factors in transgenic tobacco plants (Niggeweg *et al*., 2000a; Pontier *et al*., 2001) and by chromatin immunoprecipitation assays (Johnson *et al*., 2001; Johnson *et al*., 2003). To elucidate the mechanism by which SA regulates the defense response against pathogens, several mutant genetic screens have been carried out leading to the identification of different alleles all belonging to the same locus: npr1 (nonexpressor of PR genes 1) (Delaney *et al*., 1995; Glazebrook *et al*., 1996; Cao *et al*., 1997). NPR1 is a co-activator of *PR* genes expression and is essential to induce SAR after pathogen attacks (Cao *et al*., 1994). NPR1 interacts with several TGA transcription factors that bind the promoter of SA induced genes that possess *as-1*-like elements (Zhang *et al*., 1999; Despres *et al*., 2000; Niggeweg *et al*., 2000b; Fan and Dong, 2002; Johnson *et al*., 2003; Zhang *et al*., 2003). SA leads to NPR1 protein activation by promoting the reduction of two of its cysteines and stimulating a transition from an oligomeric to a monomeric form (Mou *et al*., 2003). The monomeric and reduced form of NPR1 is then translocated from the cytosol to the nucleus where it activates *PR1* gene expression (Kinkema *et al*., 2000). NPR1 redox modification and subsequent translocation to the nucleus are both necessary for SAR (Mou *et al*., 2003). Therefore, well-controlled redox cellular balance immediately after pathogen infections, seems to be crucial to activate the defense response.

We previously characterized the time course for SA activation of two immediate early genes with antioxidant and detoxifying functions in *Arabidopsis*: *GST6*, coding for a glutathione

S-transferase, and *IEGT*, coding for a glycosyltransferase (Uquillas *et al*., 2004). As proposed (Dong, 2004), the whole antioxidant activity within the cell should increase before NPR1 activation to generate the proper redox environment that will reduce and activate NPR1. Therefore, it would be expected that at least some of the antioxidant enzymes early induced by SA, might be independent of NPR1. Consistent with this idea, both genes, *GST6* and *IEGT*, were induced by SA independently of NPR1 (Uquillas *et al*., 2004). In an attempt to better characterize the mechanism by which SA regulates the expression of immediate early genes and to understand the first events occurring after SA accumulation, which might be crucial to trigger HR and SAR, we decided to search for other genes early induced by SA.

In this paper, we report the identification of a group of *Arabidopsis* genes, which are up- or down-regulated by SA with an early kinetics, using the cDNA-AFLP technique. We identified 12 genes induced by SA after 0.5–2.5 h of treatment. We also demonstrated that 50% of these genes require NPR1 for their induction. Finally, bioinformatic analysis showed that the *as-1*-like motif is over-represented only in the promoter of the genes that do not require NPR1 for their induction by SA.

Materials and methods

Plant growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) was used in this study. *npr1-1* mutant (Col-0) was obtained from Xinnian Dong (Department of Biology, Duke University) (Cao *et al*., 1994). Surface-sterilized seeds were germinated on solid MS medium containing 15 g l^{-1} sucrose and kept for 48 h in the dark at 4 °C for stratification. Seedlings were grown *in vitro* for 15 days in a growth chamber (22 ± 2 °C, 16 h light, 61 μmol m^{-2} s^{-1}) and maintained in darkness during the last 12 h before the experiment. All samples used in this work were collected at the same time of the day, 9:00 am, from plants kept in exactly the same conditions.

For cDNA-AFLP analysis 30–40 seedlings (400 mg fresh weight) were used for each treatment, while for Northern analysis 10–15 seedlings were used for each treatment. To minimize the effect of wounding and root breakage; seedlings were gently removed from the solid medium by taking hold of the basal part of the stem with a thin forceps and delicate pulling. Plants were then carefully placed, root side down, in a Petri dish containing 25 ml of the corresponding concentration of SA in water. Stock solution of SA (Sigma or Riedel-deHaën) was freshly prepared in H_2O. Control treatments were performed in 25 ml of water. A control sample without treatment was also included. Treatments were carried out for 15, 30, 60, 150 and 300 min in a growth chamber under constant temperature (22–25 °C) and light (61 μmol m^{-2} s^{-1}). Immediately after treatments, seedlings were frozen in liquid nitrogen and stored at −70 °C.

RNA isolation and cDNA synthesis

Total RNA was extracted from frozen plant tissue samples using the TRIzol® Reagent (Invitrogen), according to the manufacturer's protocol. First strand of the cDNA was synthesized from 10 μg total RNA by incubation in the following reaction mix: 700 ng biotinylated d(T)25 oligonucleotide, 200 U reverse transcriptase (Superscript II), 20 nmol dNTPs and buffer (25 mM Tris–HCl pH 8.3, 37 mM KCl, 1.5 mM $MgCl_2$) in a final volume of 40 μl for 2 h at 42 °C. For the second strand synthesis, the following components were added to the previous reaction mixture: 15 U *E. coli* DNA ligase, 50 U *E. coli* DNA polymerase I, 1.6 U RNase H, 30 nmol dNTPs, 3.7 mM DTT and *E. coli* ligase buffer (18.8 mM Tris–HCl pH 7.0, 4.6 mM $MgCl_2$, 90.6 mM KCl and 150 μM NAD+, 10 mM $(NH4)_2SO_4$) in a final volume of 160 μl. This mixture was first incubated for 1 h at 12 °C and then for 1 h at 22 °C. Double stranded cDNAs were then purified by using the Qiaquick PCR purification kit (Qiagen).

cDNA-AFLP analysis

cDNA-AFLP analysis was performed basically as described by Bachem *et al*. (1996) with some modifications especially designed for *Arabidopsis* (Breyne *et al*., 2002). Briefly, the procedure was carried out as follows: In the first step, transcript derived fragments (TDFs) were generated by digestion of the cDNAs with a rare-cutting enzyme (*BstY*I) and a frequent-cutting enzyme (*Mse*I). Biotinylated cDNAs (500 ng) were first digested with *BstY*I (10 U) in a final volume of

40 μl of RL buffer (restriction–ligation buffer: 10 mM Tris–Hac, pH 7.5, 50 mM K-acetate, 5 mM DTT, 5 ng μl^{-1} BSA) for 2 h at 60 °C. The 3́ ends of the cDNA fragments were then immobilized to paramagnetic beads Dynabeads® M-280 Streptavidin (Dynal) following the manufacturer's instructions. Bead-coupled cDNA fragments were then digested with *Mse*I (10 U) in a final volume of 40 μl of RL buffer for 2 h at 37 °C. Liberated cDNA fragments were removed from the paramagnetic beads. Adaptors (named: *BstY*I and *Mse*I adaptors) carrying complementary sticky ends for *BstY*I and *Mse*I restriction cuts, were then ligated to the digested cDNA fragments. Prior to ligation, both adaptors were prepared by heating a mix of two complementary oligonucleotides at 65 °C for 10 min followed by a slow cooling at room temperature (see below for oligonucleotide sequences). For the ligation reaction, the digestion mix was supplemented with 10 μl of RL buffer containing the two annealed adaptors (5 pmol *BstY*I adaptor and 50 pmol *Mse*I adaptor), 1 U T4 DNA ligase and 1 mM ATP, and the reaction was carried out for 3 h at 37 °C.

TDFs were then subjected to two rounds of amplifications. Pre-amplification reaction was performed using the adaptor-ligated cDNA fragments as a template and oligonucleotides complementary to the corresponding adaptors as primers. Conditions for pre-amplification reactions were as follows: adaptor-ligated cDNA fragments (1/4 volume of ligation mix), primers (75 ng each), 1 U of *Taq* DNA polymerase (Promega), 0.2 mM dNTPs and PCR buffer (10 mM Tris–HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl) in a final volume of 50 μl. The reaction was carried out for 25 cycles (94 °C, 30 s; 56 °C, 60 s; 72 °C, 60 s).

In a second round of amplification, independent subpopulations of the pre-amplified cDNA fragments were selectively reamplified. The selective primers used during this step were identical to the pre-amplification primers but extended by two nucleotides at the 3' end (in the case of the *BstY*I primer), or one nucleotide (in the case of the *Mse*I primer). For each combination of primers, different extended nucleotides were used providing a total of 128 different combinations. To visualize the reaction products by autoradiography, the *BstY*I selective primer was end-labeled with [γ^{33}P]ATP and T4 polynucleotide kinase (Gibco), according to the provider's protocol. Conditions for selective amplification reactions were as follows: cDNA fragments (5 μl of a 1/600 dilution of the pre-amplification mix), 5 ng ^{33}P-labeled *BstY*I selective primer, 30 ng *Mse*I selective primer, 0.6 U Ampli-Taq Gold DNA polymerase (Applied Biosystems), 0.2 mM dNTPs and PCR buffer in a final volume of 20 μl, with 13 cycle touchdown PCR (94 °C, 30 s; 65 °C (−0.7 °C/cycle), 60 s; 72 °C, 60 s) followed by 23 cycle PCR (94 °C, 30 s; 56 °C, 30 s; 72 °C, 60 s). Selective amplification products were then resolved in a 6% polyacrylamide sequencing gel at 100 Watts for approximately 3 h and detected by autoradiography. In this gel, the largest visible products are about 1500 bp in size and the smaller ones about 100 bp. In this size window, an average of 50 bands can be scored for each primer combination.

Oligonucleotides used as adaptors, and primers for cDNA-AFLP analyses are given below:

*BstY*I adaptor forward: 5′-CTCGTAGACTGCG-TAGT-3′
*BstY*I adaptor reverse: 3′-CATCTGACGCAT-CACTAG-5′
*BstY*I pre-amplification primer: 5́-GACTGCG-TAGTGATC(C/T)-3′
*BstY*I selective primer: 5′-GACTGCGTAGT-GATC(C/T)NN-3′
*Mse*I adaptor forward: 5′-GACGATGAGTCCT-GAG-3′
*Mse*I adaptor reverse: 3′-TACTCAGGACT-CAT-5′
*Mse*I pre-amplification primer: 5′-GATGAGTC-CTGAGTAA-3′
*Mse*I selective primer: 5′-GATGAGTCCTGAG-TAAN-3′

All primers were HPLC or gel purified.

Two biologically independent samples were analyzed by cDNA-AFLP. Both samples were compared using the same sets of five different primer combinations. Each combination of primers gave essentially the same TDFs pattern in both samples, supporting the technical reproducibility.

Isolation and sequencing of TDFs

Gel slices containing TDFs from differentially expressed genes, were identified by alignment with the autoradiogram. Gel slices were excised from the

polyacrylamide gel, crushed with a micropipette tip, and incubated in 100 μl of TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, pH 8) for 1 h at room temperature with occasional vortexing. The eluted DNA fragments were then reamplified by PCR using 2.5 μl of the eluted sample as template with the same combination of primers used during the second round of amplification, using the conditions described for the pre-amplification reactions. The resulting PCR products were checked on 1.2% agarose gels, purified with a Qiaquick PCR purification kit (Qiagen) and sequenced with the selective *BstYI* primer.

To identify the corresponding genes, nucleotide sequences from the amplified DNA fragments were analyzed against the *Arabidopsis* genome (GenBank database) by using the BLAST sequence alignment program available at NCBI (www.ncbi.nlm.nih.gov).

Northern blot analysis

For Northern blot analysis, samples containing 20 μg total RNA were separated on formaldehyde-agarose gels. After RNA-transfer onto nylon membranes (Immobilon-Ny+, Millipore), filters were hybridized overnight with the ^{32}P-labeled probe (about 20×10^6 cpm) in ULTRAhyb solution (Ambion) at 48 °C. Then, membranes were washed twice at 52 °C in 2× SSC, 0.1% SDS for 10 min. Radioactivity was detected with either a Phosphorimager (Cyclone, Storage Phosphor screen, Packard Bioscience Company) or by exposing to an autoradiography film for 24 h.

Gene specific DNA probes were obtained by PCR, using cDNA from SA-treated seedlings as the template, and specific primers designed from the gene sequence. Every probe was designed to specifically amplify the target gene and was verified by sequencing. Sequences for the gene specific primers, as well as the size of the corresponding PCR amplified fragment, are described in Table 1. Probes were gel purified and labeled by PCR using 1 U of *Taq* DNA polymerase (Promega), gene specific primers (5 pmol each), α^{32}P-dCTP (3.3 μl, 33 mCi), dNTPs (33 pmol each except dCTP), and PCR buffer into a final volume of 20 μl. The PCR was carried out for 36 cycles (94 °C, 30 s; variable, 75 s; 72 °C, 90 s) and purified through a micro bio-spin 6 column (BioRad).

Promoter analysis

To search for common promoter elements present in the genes co-regulated by SA, we considered 800 bp upstream from the start codon for each gene. Promoter sequences were retrieved from TAIR database (*The Arabidopsis Information Resource*, http://www.arabidopsis.org). MotifSampler software was used for subsequent sequence analysis (http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html) (Thijs *et al.*, 2002). The background distribution of nucleotides was predicted by using a 3rd order model of pre-compiled intergenic regions from *Arabidopsis* (Thijs *et al.*, 2001). Because of the stochastic searching method used, we combined input parameters to avoid a parameter chosen bias. Different analyses were performed using motif lengths (w) of 5, 8, 16 and 20 bp. For each run, the number of different motifs per sequence was fixed to a value of 2. The prior probability of finding one motif was fixed to 0.2 and the maximum number of copies per sequence for a given motif (Cmax) was unset. Both DNA strands were always scanned. Independent analyses were performed varying the over-lapping parameter to a value of 0, 1, 2, 3 and 4 nucleotides between motifs. The algorithm was iterated 4000 times for each combination of parameters to ensure convergence to the most optimal solutions. We merged all the independent outputs for each motif length and the 10 highest scored motifs were selected and ranked according to their log-likelihood scores (Ll). Motifs were ranked using MotifRanking, an application especially designed for that purpose which take into account the Kullback–Leiber distance to discriminate how similar the motifs matrices are (http://www.esat.kuleuven.ac.be/~thijs/download.html) (Thijs *et al.*, 2002). For MotifRanking process we fixed a threshold value of 0.4 where the distances between motifs lower than threshold were taken as similar. Finally, we searched the PLACE (Database of Plant *Cis*-acting Regulatory DNA elements, http://www.dna.affrc.go.jp/PLACE/) (Higo *et al.*, 1999) and TRANSFAC (The Transcription Factor DataBase, http://www.cbil.upenn.edu/tess/) (Wingender *et al.*, 1996) databases to

Table 1. Primers used to obtain probes for Northern blot analysis.

Gene	AGI number	Forward primer	Reverse primer
GST25	At2g29420	GATTCCGGTTCTTGTTCATAATG	AACGCCCCAAAGTCGCCAC
GT	At2g43820	GAAATGGAGAGACTTGGC	CAACAAGAAGCAATATGGGG
UGT1	At1g05560	GTCACTTGTGTCTCCGTCTT	CCATGAAATGAGTGTAATAGATG
UBQ10	At4g05320	GGCAGAACTCTTGCTGACTA	GCTCTCTACCTCCAAAGTGATA
DOX1	At3g01420	CGAAGACATCTTCACCAACAA	CGATAAACTTTTTCCTTCCTAATA
ANK	At5g54610	GGCAATGGAACTGATGATTCTA	TGAGCTGCTCGTATTTATCGTT
WAK1	At1g21250	CGTGTACGCAGCTGGTGAA	GTTGTTGGCGGAAAGAGATAAA
RLK	At2g37710	CGGCACCGTTTCATCCTTCT	GGCGAGCTTTGCACAGACTT
CPK31	At4g04695	GGTCAAAGCTATCGACTTCGG	CCATGATTCGCTGTCAACGTC
EP1	At4g23170	GCTGTGTTCTATTACGAGGAGTGT	GTTCGGTCAATTCGGTCTTTA
Myb	At3g11280	ACCGGTTCGTTGTTCAAGAGAT	GGGAGAAGAAGCTGCAGGATAA
OPR1	At1g76680	GATCTGGCATGTTGGCCGCG	CGATCTCCTTAGCAACTGCATC
IEGT35	At4g34135	CCATAGCAGAAGTAAGATGAAAGC	GAATCTTGAACCATTGATTTTCTCC
IEGT38	At4g34138	GAGGAAACGGGCCAAGGAG	CCATAACTTGCAATACATTCAAC
GST6	At2g47730	CCCCGTCGATATGAGAGC	GAGAGAGGGTCACTACTGCTTC
Actin	At3g53750	GCTATGTATGTCGCCATTCAAGC	CATCATATTCTGCCTTTGC(A/G)ATCC

look for known promoter motifs and transcription factors already described in plants, that matched the motifs obtained from our analysis.

For the manual search of stress-related *cis*-elements, we directly looked in our genes for 15 motifs previously described (Mahalingam *et al.*, 2003). Both, the forward and the reverse-complement sequences were considered, and the following motifs were analyzed: W-box (TTGACY), G-box (CACGTG), H-box (CCTACC), over-lapping TGA (TGACG) and SA-inducible (TGACGT) motifs, ABA-responsive element (BACGTGKM), two ethylene-related motifs (GCC-box (GCC GCC) and EIN_3 motif (GGATGTA), drought-responsive element DRE (DRC CGACNW), heat shock element HSE (CTNG AANNTTCNA), Myc-element (CACATG) and four different Myb motifs ($AtMyb_1$, MTC CWACC; $AtMyb_2$, TA-ACSGTT; AtMyb3, TAACTAAC; AtMyb4, AMCWAMC).

Results

Analysis of gene expression using cDNA-AFLP

We previously characterized the induction kinetics of two early SA-activated genes: *GST6* and *IEGT* (Uquillas *et al.*, 2004). To better understand the role of SA during early events of the stress response, we decided to look for other genes activated by SA with similar kinetics. For this purpose, *Arabidopsis* seedlings were treated with 0.5 mM SA (or water as a control) for 15, 30, 60, 150 and 300 min. A control sample without treatment (time 0), was included to monitor the plant basal status. RNA samples were then processed according to the cDNA-AFLP protocol described in 'Materials and methods.' To be able to cover a broad spectrum of the genome, two key strategies were followed: (1) we selected two restriction enzymes, *BstY*I and *Mse*I, which render an informative DNA tag for as many genes as possible (around 60% of the expressed RNAs described for *Arabidopsis* (Breyne *et al.*, 2002); and (2) 128 different combinations of primers were used during the second round of cDNA amplification. These parameters allowed us to visualize about 5680 TDFs between 100 and 1500 bp in size (Figure 1A).

From the total TDFs visualized in our screening, we selected 59 interesting targets: 39 that increased in abundance (up-regulated), and 20 that decreased in abundance (down-regulated) after SA treatments (See Figure 1 for examples). For the TDFs that increased in abundance we only considered those that showed a pattern of increase during at least two consecutive time points. This feature matches the characteristic expression pattern of genes early activated by SA (Uquillas *et al.*, 2004, see also *IEGT35*, *IEGT38* and *GST6* in Figure 2B).

As we have shown previously, genes early induced by SA can also be slightly induced by the manipulation conditions (Uquillas *et al.*, 2004). For this reason, and in order to avoid false positives due to circadian changes, we included control samples (water treatment) for each time

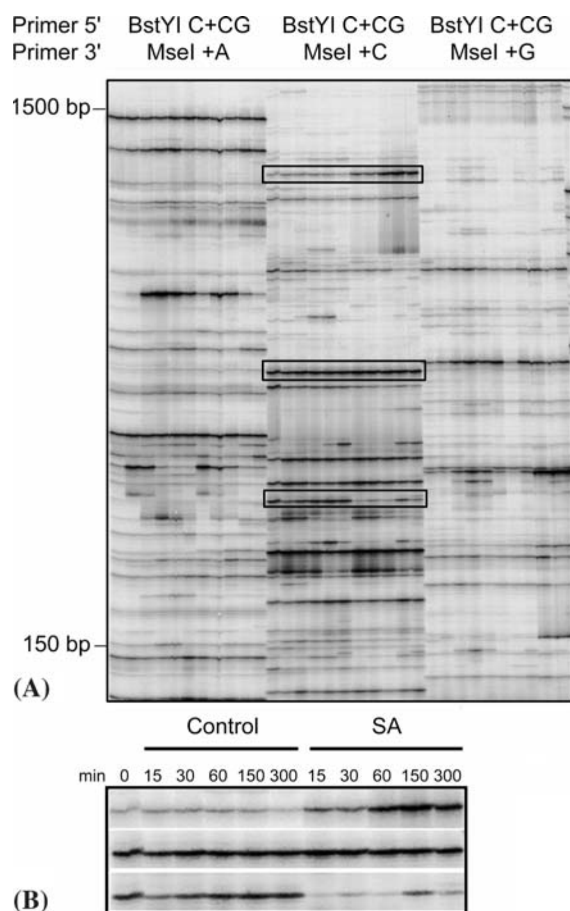

Figure 1. Example of the results obtained with the cDNA-AFLP analysis. (A) Transcript derived fragments (TDF) resulting from selective amplification using three combinations of primers, which are indicated in the top of the figure. Each row corresponds to a single time course point, after treatment with or without 0.5 mM SA (see 'Material and methods' for details). The boxes highlight examples of TDFs that were induced, unaffected or repressed by SA. Molecular markers in bp are shown in the left of the figure. (B) The same TDFs selected in part A were enlarged; the treatment (control (C) or SA), and the time of incubation are shown.

point, and TDFs were selected only when SA inductions were clearly superior to the corresponding control treatments.

Figure 1A shows an example of cDNA-AFLP fingerprints obtained with three different combinations of primers. Each primer combination was used with the 11 samples included in the screening (1 sample without any treatment, 5 samples without SA, and 5 samples with SA). Expression patterns for a transiently increasing TDF (up-regulated), a constitutively accumulating TDF (constitutive), and a steadily decreasing TDF (down-regulated), are indicated in Figure 1A. The same selected TDFs shown in Figure 1A are enlarged in Figure 1B and treatment conditions for each sample are shown.

Genes early regulated by SA identified by cDNA-AFLP

From the 59 differentially expressed TDFs selected from our screening, good quality DNA sequences (between 270 and 870 bp) were obtained for only 52 of them, while the other 7 TDFs were a mixture of PCR products and hence were not further analyzed. Sequences obtained were compared against the whole *Arabidopsis* genome using the BLAST program. Assignment of a TDF to an *Arabidopsis* gene was made when 100% identity between both nucleotide sequences was found. Eight out of 52 TDFs were discarded because they did not meet this sequence identity criterion. In addition, 3 TDFs obtained from different combination of primers, corresponded to genes already represented by other TDFs. This analysis allowed us to identify a total of 41 genes regulated by SA with an early kinetics: 21 up-regulated and 20 down-regulated.

The predicted expressed transcripts for each of the 41 genes were analyzed *in silico* to look for *BstY*I and *Mse*I restriction sites able to generate a 100–1500 bp long fragment within their sequence, a condition imposed by our assay. One up-regulated gene, which failed this test, was eliminated. Finally, we ended up with 20 putative up-regulated and 20 putative down-regulated genes.

Because we are particularly interested in genes induced by SA, we focused our effort on the up-regulated genes. Therefore, besides their identification, no further characterization was done for down-regulated genes.

All 20 putative up-regulated genes were tested by Northern blot to confirm the cDNA-AFLP results. We were unable to detect the transcripts corresponding to 4 genes: At4g35630, At1g53710, At1g14890 and At3g10930, probably due to low levels of expression. We are not certain whether these genes are expressed at very low levels or they require different conditions for their detection. The remaining 16 genes were analyzed in triplicate

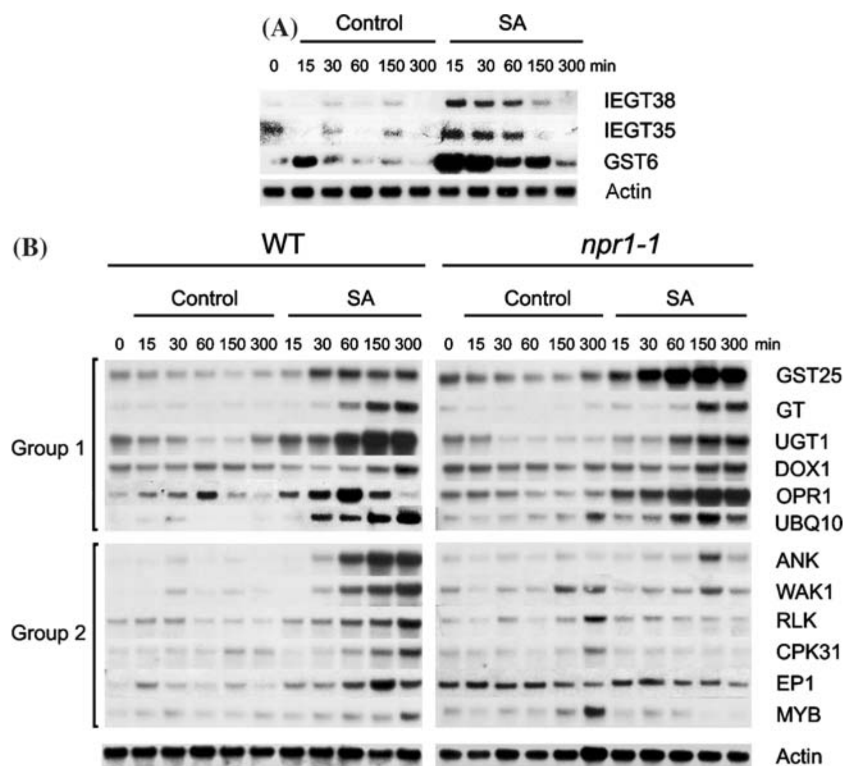

Figure 2. Kinetics of expression for genes early induced by SA. (A) Northern blot analysis for genes previously described as early induced by SA. (B) Northern blot analysis for genes isolated during the AFLP-TP screening. Genes were separated in two groups according to their requirement of NPR1 for their expression. Fifteen-day-old Col-0 (WT) and *npr1-1* mutant plants were treated with or without 0.5 mM SA during the periods of time shown at the top of the figure. Twenty micrograms of total RNA were loaded per lane, and the blots were sequentially hybridized with specific probes for the genes mentioned in the figure. Actin was used as a loading control.

using independent biological replicates and their kinetics of induction by SA was confirmed for 12 of them.

Figure 2A shows the expression kinetics for *GST6*, *IEGT35* and *IEGT38* as examples of known genes early activated by SA. *IEGT35* and *IEGT38* were originally incorrectly annotated and because they share a high percentage of sequence similarity, in our previous report both genes were detected together as *IEGT* (Uquillas *et al*., 2004). In this work, we designed specific probes for each gene and we confirmed that both transcripts are regulated by SA with the same kinetics.

Figure 2B (left panel) shows the induction kinetics obtained by Northern blot for each of the 12 confirmed genes in wild type plants. All the transcripts encoding for the new SA-regulated genes reached their pick of induction after 60–150 min of treatment; these results confirmed that the expression kinetic assays revealed by cDNA-AFLP are highly reliable.

Functional classifications and general information about the genes identified in Figure 2B are summarized in Table 2. When available, we also included in this table information from microarray experiments for each of these genes. All the above information was mainly obtained from TAIR web page (http://www.arabidopsis.org).

All confirmed genes can be classified in two general functional groups: (1) genes involved in detoxification, oxidative stress balance or cellular damage repair (*GST25*, *GT*, *UGT1*, *DOX1*, *OPR1* and *UBQ10*), and (2) genes involved in signal transduction (*ANK*, *WAK1*, *RLK*, *CPK31*, *EP1*, and *MYB*).

All genes previously described as early induced by SA (Figure 2A): *GST6* (glutathione *S*-transferase), *IEGT35* and *IEGT38* (glycosyltransferases)

Table 2. Genes early up-regulated by SA.

Gene keyword	AGI number	Gene description	Pick time (min)	Microarray**	Reference
Group 1: Detoxification, cell damage repair					
GST25	At2g29420	Glutathione *S*-transferase belonging to the tau family of GSTs (AtGSTU7). Predicted to be localized in the cytosol. Detoxification	150	NA*	TAIR (Wagner *et al.*, 2002)
GT	At2g43820	UDP-Glycosyl transferase (UGT74F2). Involved in the tryptophan synthesis pathway. Glycosylate SA *in vitro*. Metabolism and/or detoxification	150	Induced by virus, fungus and bacteria (virulent and avirulent).	TAIR (Quiel and Bender, 2003) (Lim *et al.*, 2002)
UGT1	At1g05560	UDP-Glycosyl transferase (UGT75B1). Co-purify with the callose synthase complex. Localize in the phramoplast. Metabolism and/or detoxification	150	Induced by virus	TAIR
DOX1	At3g01420	Pathogen-responsive alpha-dioxygenase. Fatty acid β-oxidation. Induced by oxidative stress. Previously reported as induced by SA	150	Induced by compatible and incompatible bacterial infections	TAIR (de Leon *et al.*, 2002)
OPR1	At1g76680	12-oxophytodienoate reductase. Involved in modification of lipid. Up-regulated by senescence and jasmonic acid. Involved in detoxification	60–150	Induced by fungus and bacteria	TAIR (He *et al.*, 2002)
UBQ10	At4g05320	Polyubiquitin (SEN3, senescence-associated protein 3). Involved in protein degradation. Induced by senescence	150–300	Induced by auxin, ethylene and fungus infection	TAIR (Park *et al.*, 1998)
Group 2: Signal transduction					
ANK	At5g54610	Belongs to the ankyrin repeat protein family	150	NA	TAIR
WAK1	At1g21250	Cell wall-associated kinase, may function as a signaling receptor of extracellular components. Induced by pathogens and SA	150	NA	TAIR (He *et al.*, 1998)
RLK	At2g37710	Similar to receptor lectin kinase 3. Has a lectin kinase domain	150–300	Expression reduced in NahG plants	TAIR
CPK31	At4g04695	Putative calcium-dependent protein kinase	150–300	NA	TAIR
EP1	At4g23170	Unknown expressed protein. Similar to receptor-like protein kinase 4 and 5. Probably localize to the endomembrane system	150	NA	TAIR
MYB	At3g11280	myb family transcription factor. Has a DNA binding domain	150–300	Induced by auxin and ERF transcription factors that regulate PR gene expression	TAIR

NA*: No relevant information available at TAIR database. Microarray**: We only included information coming from results where the conditions of the experiment were clearly explained and significant induction occurred in at least two replicates.

(Chen and Singh, 1999; Uquillas *et al.*, 2004) can also be added to Group 1.

Genes with detoxification function have been long known to be induced by a broad spectrum of pathogens during the defense response (Maleck *et al.*, 2000). In agreement with this idea, all the genes for which we found reliable information in microarray experiments, are also induced by at least one pathogen (See Table 2. Genes *GT*, *UGT1*, *DOX1*, *UBQ10*).

Interestingly, Group 2 integrated by genes involved in signal transduction, is highly repre-

sented by genes with predicted kinase activity: 4 out of 6 genes. Not much is known about any of these genes besides WAK1, that was previously described as a cell wall-associated kinase induced by pathogens and SA (He *et al.*, 1998).

Requirement of NPR1 for the SA induction of early activated genes

NPR1 is a well-known positive regulator of the SA signaling pathway (Cao *et al.*, 1997). We previously showed that NPR1 is not required for the induction of the SA early activated genes, *GST6* and *IEGT* (*IEGT35* and *IEGT38*) (Uquillas *et al.*, 2004). Therefore, we decided to test whether the 12 new SA early activated genes were also independent of NPR1 for their induction by SA.

To assess this point, *npr1-1* mutant plants (likely a null mutation (Dong, 2004)) were grown in parallel with wild type plants, and the kinetics of induction by SA of each gene was analyzed by Northern blot in both genetic backgrounds. Samples from both backgrounds were always prepared in parallel and hybridized together.

As shown in the right panel of Figure 2B, only 6 out of 12 genes were independent of NPR1 for their induction by SA. Interestingly, all the genes that did not require NPR1 belonged to Group 1 that might function in detoxification. On the other side, all genes from Group 2, with functions related to signal transduction processes, required a functional NPR1 protein to respond to SA.

EP1, a gene with similarity to receptor-like kinase 4 and 5, represents a special case. EP1 has a low level of expression in the absence of treatment (see time 0 and control treatments of WT plants, Figure 3), but contrary to its response in wild type plants, it became insensitive to SA and constitutively expressed in *npr1* mutant plants even in the absence of SA (see all the samples of *npr1-1* plants, Figure 2B). In this case, NPR1 seems to be responsible not only for the SA-dependent induction of this gene, but also for the constitutive repression of its basal expression.

It should be mentioned that, although increases in the transcripts levels for all genes described here can be detected after 30–150 min of treatment with SA, we found differences among experiments at the time point when a particular gene reached its peak of induction. We believe that the kinetics of response to SA, and probably to stress, is strongly sensitive to differences in the basal physiological status of the plants.

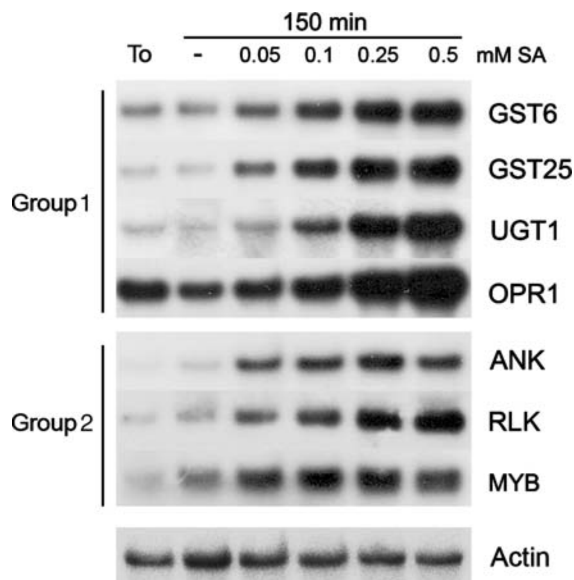

Figure 3. Effect of SA concentration in the induction of genes from Groups 1 and 2. Fifteen days old Col-0 plants were treated for 150 min with the concentrations of SA indicated at the top of the figure (from 0.05 to 0.5 mM). To, untreated plants; -, plants treated with water for 150 min as a control. Twenty micrograms of total RNA were loaded per lane, and the blots were sequentially hybridized with specific probes for the genes mentioned in the figure. Actin was used as a loading control.

Even when treatment with 0.5 mM SA is a condition broadly used in *Arabidopsis* plants to test genes induced by SA, we wondered if smaller concentrations, that might be closer to physiological conditions, were also able to induce our genes. For this purpose, we selected 3 genes belonging to Group 1, 3 genes belonging to Group 2, and *GST6* as an example of a previously known gene. Northern blot analyses were performed for these 7 genes after treating plants during 150 min with concentrations of SA ranging from 0.05 to 0.5 mM. As shown in Figure 3, all genes were induced by SA in a dose-dependent manner. More importantly, most genes were induced by concentrations of SA as low as 0.05 mM. These results strongly suggest that the regulatory effect shown here for SA, is the consequence of a physiological response and not due to a toxic effect produced by the chemical treatment.

In silico *search for putative promoter elements*

Given that the 12 SA up-regulated genes were divided in two groups based on their dependence on NPR1, the next step was to examine if they had common promoter motifs that might be responsible for their concerted activation. Analysis of genes from Group 1 (NPR1-independent) and Group 2 (NPR1-dependent) allowed us to look for differences that might explain their dissimilar NPR1 requirement. *GST6*, *IEGT35* and *IEGT38* were also included in Group 1.

The *in silico* promoter analysis was performed using the software MotifSampler (Thijs *et al.*, 2002). This software allows to search for over-represented motifs in a number of promoters of interest by comparing them against a large collection of promoters carefully selected from the *Arabidopsis* genome (background sequences) (Thijs *et al.*, 2001). Over-represented motifs are then ranked according to their log-likelihood score, which depends on the conservation of the motifs and on the number of times each motif is found in the input sequences. Table 3 shows the 10 best ranked motifs for each group of genes resulting from the 8 bp motifs search. To analyze if these motifs 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). Results from this analysis are also included in Table 3. Interestingly, for NPR1-independent genes, 4 out of the 10 best ranked 8 bp-motifs obtained, are perfect matches (100% identity in 8 bp) to the *as-1*-like element (also named *ocs*-like) described in *GST6* promoter (Chen *et al.*, 1996), while another one is a perfect match to an ABA-responsive element. In contrast, for NPR1-dependent genes, we did not find motifs that perfectly match to any known plant regulatory sequence. In this case, only one motif showed partial match to the *as-1*-like element found in the *GST6* promoter (see Table 3).

Considering that the *as-1*-like elements found in genes early activated by SA was originally described as a 20 bp-sequences containing two imperfect palindromes spaced by four nucleotides (perfect palindromic *as-1*: TGACGTCAnnnnT-GACGTCA) (Krawczyk *et al.*, 2002), we decided to search for this element, extending the motif length to 20 bp. Consistently, 8 out of the 10 highest scored 20 bp-motifs obtained from NPR1-independent genes, can be aligned with the 'perfect' *as-1* (Figure 4A). Part of the palindromic cores corresponding to the most conserved positions of *as-1*, were detected in these motifs (nucleotides in bold in Figure 4A). In contrast, we did not find correspondence with *as-1* for any of the 10 best scored 20 bp-motifs obtained for NPR1-dependent genes (data not shown). Using as a reference the conserved positions in the 20 bp-motifs of the 'perfect' *as-1* (underlined nucleotides in Figure 4A), we searched for this pattern in all the promoters under study. We found *as-1*-like elements in 6 out of 9 NPR1-independent genes (Figure 4B). In contrast, this pattern was not found in any of the promoters of NPR1-dependent genes.

To complete the *in silico* analysis, we also performed, in both groups of promoters, a search for previously described stress-related *cis*-elements (Mahalingam *et al.*, 2003). The main difference we found between both groups of promoters was the frequency of occurrence of the TGA/SARE motif (TGACG/TGACGT), which partially over-laps one of the palindromic cores of *as*-1. Consistently with our results obtained from the MotifSampler analysis, the TGA/SARE motif was found more frequently in promoters of NPR1-independent genes (1.8 instances/promoter) than in those of NPR1-dependent genes (0.8 instances/promoter).

In summary, we found a consensus *as-1*-like element over-represented in promoters of the NPR1-independent genes and not in NPR1-dependent genes.

Discussion

In the present work, we described 12 new genes early induced by SA. We demonstrated that 6 of these genes, all involved in functions related to detoxification or cellular damage repair, do not require NPR1 for their SA-dependent induction. On the other hand, the remaining 6 genes, with functions related to signal transduction processes, do require NPR1. Finally, careful analysis of the promoter of these two groups of genes allowed us to propose that the *as-1* sequence may only play an important role in the regulation of genes belonging to Group 1, which are independent of NPR1 for their induction by SA.

Table 3. In silico promoter analysis of genes early up-regulated by SA.

Motif Sampler analysis[a] Consensus motif sequence	# Promoters	Instances	PLACE/TRANSFAC analysis[b] Element sequence	Factors
NPR1-independent genes				
AAATAAGG	7	8		
T(A/C)AGG(A/G)AA	7	8		
A(A/G)(A/G)GAAAG	8	12		
(C/T)AGA(T/A)TCT	7	10		
TCA(T/A)TGA(T/C)	7	10	OBF5ATGST6, ocs element found in *Arabidopsis GST6* gene promoter atcttatg**TCATTGAT**gacgacctcc	OBP5
			JASE1ATOPR1, 'JASE1' found in *Arabidopsis OPR1* gene promoter[c] **cGTCAATGAa**	
ATGACG(A/T)C	6	9	OBF5ATGST6, ocs element found in *Arabidopsis GST6* gene promoter atcttatgtcattg**ATGACGAC**ctcc	OBP5
(T/A)CCT(C/T)GTC	6	8		
GAG(A/C)CG(T/A)(A/G)	6	8	ABREBZMRAB28, ABA-responsive element (ABRE B) found in maize *RAB28* gene promoter tc**CACGTCTC**	TRAB1/VP1
(T/C)GACGA(A/C)C	6	8	OBF5ATGST6, ocs element found in *Arabidopsis GST6* gene promoter atcttatgtcattga**TGACGACC**tcc	OBP5
(C/T)(G/A)A(T/A)GACG	6	9	OBF5ATGST6, ocs element found in *Arabidopsis GST6* gene promoter atcttatgtcat**TGATGACG**acctcc	OBP5
			TGA1ANTPR1A, TGA1a binding site in tobacco (N.t.) *PR1a* gene promoter **CGTCATCG**agatgacg	TGA1a
NPR1-dependent genes				
G(A/C)AG(A/C)T(G/T)C	6	10		
G(A/G)(A/C)(A/T)ACAG	6	8		
AA(A/C)GAACA	5	6		
G(A/C)(A/T)GC(A/T)GC	5	8		
GGAACAGA	5	7		
(A/C)TGGGGCC	4	5		
ACAG(A/T)G(A/G)(C/G)	5	8		
(C/T)A(A/C)TGACT	5	7	OBF5ATGST6, ocs element found in *Arabidopsis GST6* gene promoter atcttat**GTCATTG**atgacgacctcc	OBP5
ACAGAG(C/T)(A/T)	5	7		
C(A/C)GAGA(C/G)T	5	7		

[a] Selection of the 10 highest scored 8 bp-motifs over-represented in the promoters of the 9 NPR1-independent and the 6 NPR-dependent genes identified.
[b] For NPR1-independent genes, only regulatory elements found in PLACE and TRANSFAC databases with perfect matches (100% identity in 8 bp, indicated in bold) to the consensus motifs were selected. For NPR1-dependent genes, the regulatory element with best match to a consensus motif was selected.
[c] Note that the JASE1 and JASE2 motifs described in the OPR1 promoter as senescence and JA responsive *cis*-elements (He and Gan, 2001) match perfectly with the *as-1* motif.

cDNA-AFLP analysis

The cDNA-AFLP fingerprinting technique has some advantages over other genome wide techniques for expression analysis: It does not require prior sequence information, therefore enabling identification of novel genes; it does not require expensive technology and it allows quantitative and temporal analysis. More importantly, by performing Northern blot analysis we and others have shown that cDNA-AFLP is a reliable technique. De Paepe *et al.* (2004) and Reijans *et al.* (2003) reported a good correlation between cDNA-AFLP and microarray analyses.

It should be noted that the reason why *GST6*, *IEGT35* and *IEGT38* were not recovered in our screening is because none of these genes have a *BstY*I restriction site in their sequence.

Genes early regulated by SA and their possible roles in stress defense responses

The functions predicted for genes up-regulated by SA allowed us to separate these genes into 2 main groups. (1) Genes involved in detoxification or

(A) MotifSampler analysis

Consensus motif	#Promoters	Instances
nnnCGTmwnyrnTGACGnnm*	7	13
GTCATTGnwkACGnCAymmn*	6	8
nTkACGTmknCnwnGACGwn*	6	9
wyGTCnnyrnTGACrwnmnm*	6	12
knCGTCnnCrnnGACGnnnn*	6	13
CGTCAnyrnnGACGTnnnyn*	6	14
AwrAnAGAAArnGAAwnAGA	7	10
AAwAAArwnnAnAnAACAAA	6	6
nGTCATTGnwGACGwCAynm*	5	7
GTCrTnnnwkACGwCnnnnk*	6	12
TGACGTCA-c--TGACGTCA (perfect palindrome)		

(B) *as-1*-like and TGACG-like elements

***as-1*-like elements in group 1 genes**

Gene	Position	Element	Position
IEGT38	-407	TGAC GAAT gcaa TGAC GTGA	-388
IEGT35	-104	AGAC GTAT gcaa CGAC GTCA	-85
GST6	-310	GGTC GTCA tcaa TGAC ATAA	-329
GST25	-46	TGAC GTCA ccag TGAC GAAC	-65
UGT1	-130	TGAC GTCT gcga TGAC GCTC	-149
OPR1	-45	ATAC GTCG tcaa TGAC GACC	-26
OPR1	-97	TGAC GTAT tcat TGAC GACA	-116
Consensus		DGAC GTMW bcan TGAC GHNM	

***as-1*-like elements in other genes**

Gene	Position	Element	Position
CAMV35S	-81	TGAC GTAA ggga TGAC GCAC	-62
*GNT35*Nt	-327	ATAC GTAA gcac TTAG CTAA	-346

TGACG-like motifs in *PR* genes

Gene	Position	Element	Position
PR-1a Nt	-594	TAAC GTCA tcgaga TGAC GGCC	-574
PR-1 At	-636	TCTATGAC GTAA *gtaaaatag* TGAC GTAGA	-665
		LS7 *LS6* *LS5*	

Figure 4. Over-representation of *as-1*-like elements in promoters of genes up-regulated by SA independently of NPR1 (Group 1). (A) MotifSampler analysis. The 10 best scored 20 bp-motifs, obtained from the 9 NPR1-independent genes identified, are shown. Motif, consensus motif sequence; # Promoters, number of independent promoters where the corresponding motif was found; Instances, number of times the motif was found in all promoters. Motifs that can be annealed with the *as-1* perfect palindromic sequence (shown at the bottom) are indicated with an asterik. Nucleotides with identity to *as-1* are indicated in bold. Conserved positions in the motifs annealed with the *as-1* perfect palindrome are underlined. (B) Annealing of *as-1*-like elements found in the promoters of genes from Group 1 and previously characterized genes. Positions are referred to the transcription start site. A consensus sequence from these elements was deduced. *as-1*-like elements found in the CaMV 35S promoter (Lam *et al.*, 1989) and the glutathione *S*-transferase GNT35 (Nt103-1) promoter (Droog *et al.*, 1995), are shown. TGACG-like motifs found in *Arabidopsis PR-1* (Lebel *et al.*, 1998) and tobacco *PR-1a* (Strompen *et al.*, 1998) promoters are also shown. For *Arabidopsis PR-1* promoter, responsive elements named LS5, LS6 and LS7 are indicated (Lebel *et al.*, 1998). 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.

defense against stress; and (2) genes involved in signal transduction.

The first group is composed of genes coding for gluthation *S*-transferases (*GST6* and *GST25*), glycosyltransferases (*UGT1*, *GT*, *IEGT35* and *IEGT38*), lipid metabolism (*DOX1* and *OPR1*) and protein degradation (*UBQ10*). We also included in this group three early genes that had been previously identified, GSTs (*GST6*) and GTs (*IEGT35* and *IEGT38)* (Xiang *et al.*, 1996; Uquillas *et al.*, 2004). All these biological functions have been already connected with detoxification and/or pathogen defense (see Table 2 for references).

GSTs catalyze the addition of glutathione to a variety of substrates. This covalent modification plays an important role in detoxification of xeno- and endobiotic compounds as well as in the

recovery of the cell redox balance (Marrs, 1996; Edwards *et al.*, 2000). GTs attach sugar molecules to small substrates, a chemical modification that is especially common in plant metabolism and reactions related to stress defense (Vogt and Jones, 2000). Genes belonging to both families, GSTs (around 50 family members in *Arabidopsis* (Wagner *et al.*, 2002)) and GTs (around 90 family members in *Arabidopsis* (Li *et al.*, 2001)), have been previously described as SA early induced genes (Horvath and Chua, 1996; Xiang *et al.*, 1996; Uquillas *et al.*, 2004) and are typically found upregulated in microarray experiments of plants responding to pathogen attacks or oxidative stress (Maleck *et al.*, 2000; Schenk *et al.*, 2000; Vandenabeele *et al.*, 2004). Interestingly, the gene we named *GT,* encodes for a glycosyltransferase identified as UGT74F2, that glycosylates SA *in vitro* (Lim *et al.*, 2002). A mutant of the same gene was isolated in a screening of *Arabidopsis* mutants that accumulate anthranilate, a fluorescent tryptophan precursor (Quiel and Bender, 2003). In this work, the authors demonstrated that *GT* (UGT74F2) mutant plants accumulate anthranilate *in vivo* and that the recombinant enzyme can glycosylate this substrate *in vitro*. Regretfully, they did not evaluate the effect of this mutation on the SA pathway; therefore it remains unknown whether UGT74F2 has any role in this pathway *in vivo*.

The identification in our screening of genes involved in lipid metabolism (*DOX1* and *OPR1)* is a very interesting result. Modified lipids are important signals involved in senescence and defense against pathogens and oxidative stress (Shah, 2004). DOX1 catalyzes the oxidation of fatty acids to oxylipins, molecules implicated in the plant response to pathogens, oxidative stress and senescence (de Leon *et al.*, 2002). DOX1 expression is induced by bacterial infection, SA, ROS, and senescence (de Leon *et al.*, 2002). Interestingly, after pathogen infection, the expression of this gene is confined mainly to the infection site, and *dox1* mutants develop rapidly expanding and severe necrotic lesions compared to wild type plants (de Leon *et al.*, 2002). All these evidences suggest that DOX1 might be involved in protecting plants against the oxidative stress associated with the HR (de Leon *et al.*, 2002; Shah, 2004).

OPR1 encodes a 12-oxophytodienoic acid reductase. OPR enzymes are responsible for the last steps on the synthesis of modified lipids like JA and similar molecules. The *in vivo* product of the OPR1 enzyme remains unknown, but Schaller *et al.* (2000) demonstrated that it is not involved in JA synthesis. Interestingly, *OPR1* is induced by senescence, wounding, UV-C and JA (He and Gan, 2001; He *et al.*, 2002). In the future, it would not be surprising to find that the product of OPR1 activity is also a lipidic molecule involved in one or more signaling pathways related to stress responses. In this respect, it is important to mention that several genes encoding proteins involved in lipid metabolism have been linked to the SA signaling pathway, oxidative stress response and pathogen defense. For example, *PAD4* (phytoalexin-deficient 4) and *EDS1* (enhanced disease susceptibility 1) exhibit homology to lipid acyl-hydrolases and both control SA accumulation after pathogen recognition (Feys *et al.*, 2001).

Even though UBQ10 does not have a function clearly related to detoxification or lipid metabolism (it codifies 6 in-tandem copies of ubiquitin); it is also induced by SA, pathogens and senescence (Table 2). Interestingly, UBQ10 was first named SEN3 (senescence-associated protein 3) because it was isolated in a screening for genes induced during senescence (Park *et al.*, 1998). Ubiquitin is a well-known small protein that is covalently attached to other proteins labeling them for degradation (Callis and Vierstra, 2000). Therefore, all genes that belong to Group 1 are connected in diverse ways with plant responses to pathogens or to stresses that induce cell death.

Genes early induced by SA belonging to Group 2 encode proteins involved in signal transduction functions. A remarkable point about these genes is the strong presence of putative protein kinases. Indeed, 3 out of 6 genes encode receptor like kinases (WAK1, RLK, EP1), and CPK31 is predicted to be a calcium-dependent kinase. WAK1 encodes a protein that spans the plasma membrane and contains a cytosolic kinase domain (He *et al.*, 1998). This last kinase is induced by SA and several pathogens, it is required for PR1 expression in a NPR1-dependent manner, and it is essential for plant survival to bacterial infections (He *et al.*, 1998).

We were unable to find more information about RLK, EP1 or CPK31. Nevertheless, CPK31 might represent a very interesting target for further investigation. Ca^{2+} concentration increases very

early after pathogen infection and this increase is important for the onset of the oxidative burst (Nurnberger and Scheel, 2001). A possible speculation is that CPK31 could be one of the kinases required in this early transduction pathway.

The two remaining genes of this group encode a myb transcription factor (MYB) and an ankyrin domain containing protein (ANK). Nothing is known about either one of them but ankyrin domain-containing proteins have been described in multiple steps of signaling pathways, in several organisms. NPR1 in plants, and NFκB in mammals are classical examples of this family of proteins (Cao *et al*., 1997).

From our screening, we isolated 12 genes up-regulated and 20 genes putatively down-regulated by SA. We focused our efforts in the up-regulated genes. However, even when we did not characterize down-regulated genes, they present some interesting features that deserve a comment. Fifty percent of the genes down-regulated by SA encode for proteins predicted to be localized in the chloroplast and 20% of them are involved in photosynthesis. The link between photosynthesis and pathogen or stress defenses has been proposed before. Matsumura *et al*. (2003) reported that pathogen infection inhibit the expression of several genes involved in photosynthesis before the onset of HR. In the same direction, it was shown that H_2O_2 accumulation, a well-known consequence of SA increase, repress the expression of several genes encoding for proteins involved in photosynthesis (Vandenabeele *et al*., 2004). However, further research would be needed to discern whether the effect of SA onto the chloroplast is only a secondary consequence of the cell stress imposed by SA and ROS, or if it plays an active role in the defense response to stress.

SA and NPR1

Previous work and this report have shown that NPR1 is not required for the SA-dependent induction of several genes involved in detoxification or oxidative stress balance (de Leon *et al*., 2002; Uquillas *et al*., 2004). Dong (2004) proposed that this might be due to the fact that NPR1 requires to be reduced for activation. Indeed, Mou *et al*. (2003) demonstrated that before any SA increase, NPR1 forms inactive oligomeric complexes in the cytosol. After SA increases, ROS are accumulated and detoxifying genes like GSTs are rapidly induced (Draper, 1997; Garreton *et al*., 2002; Uquillas *et al*., 2004). Subsequently, for the activation of NPR1 to occur, two of its Cys must be reduced (Mou *et al*. 2003). Once NPR1 is activated, it migrates to the nucleus and stimulates transcription of several genes like *PR1* (Kinkema *et al*., 2000), and those described in this work. All the above evidence leads to the proposition that the induction of antioxidant and detoxifying enzymes, like GSTs and GT, might help to generate the required reduced environment to activate NPR1 (Dong, 2004). This model fits perfectly with the fact that genes from Group 1, involved in detoxification, do not require NPR1 for their induction by SA. However, this model requires addition of some extra complexity to explain how genes belonging to Group 2, which are dependent on NPR1, are also early induced by SA at a time when NPR1 is apparently still inactive. The answer might be in the presence of either a very fine tuning for the timing of the redox changes inside the cell, and/or more than one mechanism of NPR1 activation.

Promoter analysis

In silico promoter analysis of the two groups of genes up-regulated by SA, allowed us to find differences that might explain their dissimilar NPR1 co-activator protein requirement. Interestingly, the *as-1*-like element (perfect consensus TGACGTCAnnnnTGACGTCA) was specifically over-represented only in genes belonging to Group 1. This led us to postulate that this element, previously characterized as a SA- and oxidative stress-responsive element, plays an important role in the regulation of cell protecting genes that are early-induced by SA independently of NPR1.

The presence of functional *as-1*-like elements and TGACG/TGACGT motifs in genes induced by SA has been extensively reported. However, the description of these elements in the literature is quite confusing. The *as-1* element was initially described in the CaMV 35S promoter as a 20-bp sequence containing two TGACG tandem motifs (Lam *et al*., 1989) that confer tissue-specific expression in the root tip (Benfey *et al*., 1990) and immediate early response to SA (Qin *et al*., 1994). Thereof, functional *as-1*-like (also named *ocs*-like) elements were found in the promoter of

other genes expressed in plants by pathogenic virus and bacteria (revised in Krawczyk *et al.*, 2002). Even more interesting, is that the same elements were also found in promoters of plant *GSTs* genes induced by SA (i.e. tobacco *GNT35* and Arabidopsis *GST6*; Xiang *et al.*, 1996; Chen and Singh, 1999). Careful alignment and analysis of these *as-1*-like elements allowed to define a 'consensus *as-1*-like element' of 20 bp containing two imperfect palindromes spaced by a conserved distance (TGACGTCAnnnnTGACGTCA) (Krawczyk *et al.*, 2002). It has been clearly established that *as-1*-like elements are able to confer immediate early response to SA, xenobiotics and other stress-related signals (Liu and Lam, 1994; Qin *et al.*, 1994; Xiang *et al.*, 1996; Garretón *et al.*, 2002), including oxidative stress (Garreton *et al.*, 2002). Substantial evidence from *in vivo* approaches indicate that the *as-1*/*ocs*-like elements are targets for TGA factors (Niggeweg *et al.*, 2000a; Johnson *et al.*, 2001; Pontier *et al.*, 2001).

On the other side, TGACG-like motifs have been found to be functional in the promoter of late SA-responsive genes such as *PR-1* from tobacco and *Arabidopsis* (Lebel *et al.*, 1998; Strompen *et al.*, 1998). The presence of two tandem repeated TGACG-like motifs in these *PR-1* promoters lead to confusingly naming these elements as '*as-1*-like.' Nevertheless, important structural and functional differences exist between these TGACG-like motifs found in *PR-1* genes and the *as-1*-like elements found in pathogens and plant *GST* genes. First, spacing between TGACG motifs in *PR-1* promoters are longer and less conserved (Krawczyk *et al.*, 2002) (see Figure 4B). Besides, functional analysis of the *Arabidopsis PR-1* promoter allowed to differentiate the two TGACG-like repeats, while one (named LS5) is involved in basal repression of the gene, the other (named LS7) is involved in SA-mediated activation (Lebel *et al.*, 1998) (see Figure 4B). LS5 and LS7 TGACG-like motifs can independently bind TGA factors *in vitro* supporting the idea of independence between them (Després *et al.*, 2000). Interestingly, the requirement of the NPR1 protein for *in vivo* interaction of *Arabidopsis PR-1* promoter with TGA factors has been demonstrated (Johnson *et al.*, 2003). In contrast, evidence suggests that both TGACG-motifs of the *as-1* element are required in concert for its activity (Benfey *et al.*, 1990; Neuhaus *et al.*, 1994). Finally, while *as-1*-like elements confer expression activated by different stress-related signals, as discussed above, *PR-1* genes are only late activated by SA (Uquillas *et al.*, 2004).

Our finding that the *as-1*-like element is over-represented in genes early-induced by SA independently of NPR1, adds further support to the idea that SA can use parallel mechanisms to activate different groups of genes. Different responsive elements, TGACG-like motifs or *as-1*-like elements, and the differential use of NPR1, can explain the differences in the kinetics of activation observed in genes regulated by SA. Whether different SA-responsive elements are *in vivo* targets for different subclasses of TGA factors remains to be elucidated.

In conclusion, in the present work we have described 12 new genes early induced by SA. These genes can be separated in two groups where Group 1 is defined by genes involved in cellular detoxification, do not require NPR1 for their induction by SA, and have at least one *as-1* element in their promoters. On the other side, Group 2 is defined by genes involved in signal transduction processes, they do require NPR1 to be induce by SA, and do not seem to have an *as-1* element in their promoters.

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