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Cloning and identification of the promoter of the tobacco *Sar8.2b* gene, a gene involved in systemic acquired resistance ☆

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Abstract

Expression of the *Sar8.2* gene family is induced by salicylic acid (SA) in tobacco during induction of systemic acquired resistance. Expression of *Sar8.2b*, one member of this 12-member family, was detected as early as 12 h after treatment with SA and was maximal 36 h after SA treatment. In *NahG* transgenic tobacco plants, benzothiadiazole and dichloroisonicotinic acid induced expression of *Sar8.2b* but SA did not, suggesting that expression of the *Sar8.2b* gene is SA-dependent. Several putative *cis*-acting elements were found in the *Sar8.2b* gene promoter region, including an *as-1* element and GT-1 and Dof binding sequences. We constructed a series of progressive deletion mutations in the *Sar8.2b* promoter region linked to the β-glucuronidase (GUS) coding region and analyzed GUS activities by stable expression in transformants of *Arabidopsis thaliana*. Deletions between −728 and −927 bp or between −351 and −197 bp of the promoter region resulted in a significant reduction in GUS activity induced by SA treatment as shown in stable transformants of *A. thaliana*. The −197 bp fragment of the promoter region was found to confer a relatively low level of GUS activity induced by SA treatment in stable expression of transformants in *A. thaliana*. The results suggest that 927 bp of the *Sar8.2b* gene promoter confers full promoter activity and that *cis*-acting elements required for high-level SA-inducible expression of the *Sar8.2b* gene may exist within the regions −728 to −927 bp and −197 to −351 bp.
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Keywords: Promoter; *Sar8.2b*; Systemic acquired resistance; Tobacco; *Nicotiana tabacum*

1. Introduction

Plants defend themselves from pathogen attack by a combination of constitutive and inducible defenses. Upon pathogen infection, specific signal transduction pathways are induced, resulting in the expression of a variety of defense responses, which include rapid programmed cell death (hypersensitive response, HR). Development of HR usually triggers a secondary defense response in the uninfected parts of the plant, which confers resistance to normally virulent pathogens (Ross, 1961; Ryals et al., 1996). This phenomenon is called systemic acquired resistance (SAR), which is characterized by a long-lasting and broad-spectrum resistance against fungal, bacterial and viral diseases (Ryals et al., 1996; Sticher et al., 1997). SAR is also activated by certain chemicals such as salicylic acid (SA), dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) (Oostendorp et al., 2001). SA has been demonstrated to be a critical signal for plant SAR, and the signaling pathway involved in SAR is called the SA-dependent signaling pathway (Dong, 1998, 2001).

Associated with the onset of SAR is the coordinate expression of a set of genes called SAR genes (Ward et al., 1991). The mechanism by which expression of SAR genes is activated during SAR is largely unknown. Studies on the promoters of several SAR genes, including tobacco *PR-1* (Uknes et al., 1993) and *PR-2d* (van de Rhee and Bol, 1993), Arabidopsis *PR-1* (Lebel et al., 1998), and parsley *PR-1* (Meier et al., 1991), have led to elucidation of the fine structures of several SAR gene promoters and the identifi-

Abbreviations: BTH, benzothiadiazole; DIG, digoxigenin; GUS, β-glucuronidase; HR, hypersensitive response; INA, dichloroisonicotinic acid; RT–PCR, reverse transcription–polymerase chain reaction; RACE, rapid amplification of cDNA ends; SA, salicylic acid; SAR, systemic acquired resistance(

☆ The nucleotide sequence of the tobacco *Sar8.2b* gene promoter has been deposited in the GenBank database under accession number U64816.

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cation of a variety of *cis*-acting elements. For example, the GCC box (also called PR box) (Hart et al., 1993; Ohme-Takagi and Shinshi, 1995; Sato et al., 1996) and TGAC-containing *cis*-acting elements *as-1* and *ocs* (Qin et al., 1994; Zhang and Singh, 1994; Guevara-Garcia et al., 1998) have been shown to be SA-responsive and thus play roles in regulation of SAR gene expression.

Sar8.2 was identified and cloned from uninfected upper leaves harvested from TMV-inoculated tobacco plants by differential screening of a cDNA library (Alexander et al., 1992). *Sar8.2* is one of the gene families whose expression is induced in tobacco during SAR induction and contains at least 12 members (Alexander et al., 1992; Moraes and Goodman, unpublished). Transgenic tobacco plants constitutively expressing one member of *Sar8.2* gene family, *Sar8.2d*, showed enhanced disease resistance against two oomycete pathogens, *Pythium torulosum* and *Phytophthora parasitica* (Alexander et al., 1993; Chen and Goodman, unpublished). Expression patterns of the *Sar8.2* gene family members differ in a stimulus-dependent manner. TMV infection or SA treatment induces a strong, long-lasting expression; while touch or wounding results in a rapid but brief induction (Moraes and Goodman, unpublished). To understand the mechanism by which expression of the *Sar8.2* genes is regulated upon SAR induction, we cloned the *Sar8.2b* gene promoter and analyzed the minimum sequences required for promoter activity by analysis in stable transformants of *Arabidopsis thaliana*. Our results indicate that expression of the *Sar8.2b* gene is SA-dependent and that *cis*-acting elements required for high-level SA-inducible expression may exist within −728 to −927 bp and −197 to −351 bp of the *Sar8.2b* gene promoter.

2. Materials and methods

2.1. Growth of tobacco plants and treatment with SAR inducers

Wild-type and the *NahG* transgenic tobacco (*Nicotiana tabacum* cv. Xanthi nc) plants were grown in Redi-earth plug and seedling mix (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) in a growth chamber under 14 h of light (27 °C) and 10 h of dark (22 °C). Light (244 μEinstein m^{-2} s^{-1} at the level of soil surface) was provided by 40 W Cool White fluorescent bulbs. Four- to 5-week-old plants were used for all experiments. For treatment with SAR inducers, one fully expanded leaf was sprayed with a fine mist of 2 mM SA (pH6.5), 0.3 mM INA, or 0.3 mM BTH (a 50% active ingredient formulation, Syngenta (Novartis) Crop Protection, Research Triangle Park, NC, USA). The wettable powder formulation for BTH (Syngenta (Novartis) Crop Protection, Research Triangle Park, NC, USA) and water were used as co-controls. The treated leaves were collected at time points indicated and frozen immediately in liquid nitrogen.

2.2. RNA extraction and Northern analysis

Total RNA was extracted using a hot-phenol method (Verwoerd et al., 1989). Briefly, leaf tissues were homogenized in liquid nitrogen and extracted in a 1:1 mixture of phenol and extraction buffer (100 mM LiCl, 100 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% SDS) at 80 °C. Half volume of chloroform was added, mixed by vortexing and centrifuged. RNA was precipitated overnight at 4 °C in 2 M LiCl. After centrifugation, the RNA was dissolved in diethyl pyrocarbonate (DEPC)-water, precipitated in 0.1 volume sodium acetate (pH 5.2) and two volumes of ethanol, and resuspended in DEPC-water.

Ten micrograms of total RNA were fractionated on 1.2% agarose-formaldehyde gel and transferred capillarily overnight to Magnagraph nylon membranes (Micron Separations Inc., Westboro, MA, USA) using 10× SSC. The RNA was fixed by baking at 80 °C for 2 h.

A 488 bp fragment of the *Sar8.2b* cDNA was amplified by RT–PCR using primers of *Sar8.2b*-1F (5′-CTC GAT ATA GCT CAT CTT TC-3′) and -1R (5′-AAA GAC TAG AAA ACA AGA T-3′) designed according to its sequence (GenBank M97359 and U64816). The PCR product was cloned and confirmed by sequencing. A 798 bp fragment of *PR-1a* cDNA (Payne et al., 1989) was cut from plasmid by digestion with *Eco*RI and gel-purified. A cloned tobacco gene *pCNT6* was used for evaluation of equal loading. The *PR-1a* cDNA and *pCNT6* fragment were labeled by the random primer DNA labeling system with digoxigenin (DIG)-11-dUTP (Boehringer Mannheim, Indianapolis, IN, USA).

Prehybridization was performed at 42 °C for 3 h in a solution containing 5× SSC, 5× Denhardt's solution, 50% formamide, 1% SDS and 100 μg/ml denatured salmon sperm DNA with rotation in Stratagene's Techne Hybridizer HB-1D (Stratagene, La Jolla, CA, USA). Probes were added to the solution at a concentration of ~10 ng/ml and hybridization was performed overnight, followed by four washings with 2× SSC, 0.1% SDS and 0.2× SSC, 0.1% SDS for 10 min each at room temperature. Detection of DIG-labeled probe was carried out by the chemiluminescence method according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, USA).

2.3. PCR amplification of the 5′ region of the tobacco Sar8.2b gene

A tobacco genomic DNA library was screened previously for isolating *Sar8.2* gene family members and clone 183 contains the *Sar8.2b* gene within a ~6 kb insert (Moraes and Goodman, unpublished; GenBank accession no. U64816). Phage DNA was extracted from clone 183 (from which the GenBank U64816 sequence was determined) and used in PCR. A vector primer EMBL-5 (5′-TGC AGA CAA ACT GCG CAA CTC GTG-3′, Manninen and Schulmann, 1993) and a *Sar8.2b*-specific primer 1323-1R (5′-AGA

CAT CTC CCT TGC ATC AGC-3′, which lies between 284 and 304 in the sequence (U64816) of the *Sar8.2b* gene) were synthesized. The GeneAmp XL PCR kit (Perkin Elmer, Foster City, CA, USA) was used following the manufacturer's instructions. The PCR reactions were incubated for 1 min at 94 °C and then run 16 cycles for 30 s at 94 °C, 30 s at 55 °C and 5 min at 72 °C, followed by 15 cycles for 30 s at 94 °C, 30 s at 55 °C and 7 min at 72 °C with a final extension for 8 min at 72 °C. The PCR product was purified using QIAQuick PCR Purification kit (Qiagen, Valencia, CA, USA) and cloned into pGEM T-Easy vector (Promega, Madison, WI, USA) by T/A cloning.

2.4. Determination of the transcriptional start site

The transcriptional start site of the *Sar8.2b* gene was mapped using Gibco-BRL's 5′-RACE system (Version 2.0, Gibco BRL, Gaithersburg, MD, USA). Total RNA was extracted from leaf samples collected 1 day after treatment with 2 mM SA and treated with RNase-free DNase to remove any trace of genomic DNA. One microgram of total RNA was used in 5′-RACE with two nested antisense *Sar8.2b* gene-specific primers, *Sar8.2b*-1R and *Sar8.2b*-2R (5′-GCC AAT GAT GTT GCC TCC TT-3′), following the manufacturer's instructions. The RACE product was cloned into pGEM T-Easy vector (Promega, Madison, WI, USA) by T/A cloning and sequenced.

2.5. DNA sequencing

Plasmids containing the target inserts were sequenced using the BigDye Sequencing Reagent. Sequencing reactions were carried out by PCR and analyzed with an ABI model 377A DNA automatic sequencer at the Biotechnology Center of the University of Wisconsin–Madison.

2.6. Construction of 5′ deletion constructs of the Sar8.2b gene promoter

The promoter fragment and part of the coding region of the *Sar8.2b* gene (the first seven N-terminal codons) was amplified using *Sar8.2b*-4F (5′-GTA TGG ATC CAT CCC TTT GTC TCC CTG TTG G-3′) and -4R (5′-GCC GCC CGG GAA GAA ATG TTT TGG AAA ACA T-3′), which contain a *Bam*HI and *Sma*I site (underlined), respectively, and the plasmid containing the cloned promoter region as template. The amplified fragment was digested with both *Bam*HI and *Sma*I and ligated into pUC18, which was digested with the same enzymes.

Nine constructs with progressive deletions from the 5′ end of the cloned *Sar8.2b* gene promoter region were made (Fig. 3). For constructs C, E, G and H, the pUC18 plasmid containing the promoter region was digested with *Bam*HI and one other restriction enzyme located at various positions in the promoter. The protruding ends were digested with S1 nuclease and the resulting blunt-ended vector fragments were self-ligated. Constructs B, D, F, I, and K were created using Promega's Erase-a-Base system (Promega, Madison, WI, USA) according to the manufacturer's recommendation. Briefly, the pUC18 plasmid containing the promoter region was digested with *Bam*HI, the site filled in with α-phosphate by Klenow DNA polymerase to render it resistant to Exonuclease III, and then digested with *Spe*I to release the susceptible end. One microgram of plasmid was used in each deletion reaction, and digestion with Exonuclease III was carried out at 30 °C using 0.5 unit of enzyme. After incubation for different time periods, one reaction was taken at each time point to give a desired deletion length at the 5′ end and then digested with S1 nuclease to remove the single-stranded region. The blunt-ended plasmids were then self-ligated and transformed into *Escherichia coli* JM109.

After verification by sequencing, all deletion constructs were released from pUC as *Sal*I–*Sma*I fragments and then cloned into binary vector pBI101.3 (Clontech, Palo Alto, CA, USA), which contains a GUS reporter gene. The full-length fragment of the cloned promoter was also released from the pUC18 plasmid containing the cloned promoter region as *Bam*HI–*Sma*I fragment and cloned into pBI101.3. These plasmids were transferred into *Agrobacterium tumefaciens* strain LAB4404 (Gibco BRL) by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Transformation of Arabidopsis thaliana and selection of transformants

A. thaliana plants of ecotype Columbia were grown in a growth chamber under 16 h of light (25 °C) and 8 h of dark (22 °C). Four- to 5-week-old flowering plants were used for transformation experiments.

A. tumefaciens was grown at 28 °C with shaking (200 rpm) in YM broth (yeast extract, 0.4 g/l; mannitol, 10.0 g/l; NaCl, 0.1 g/l; $MgSO_4 \cdot 7H_2O$, 0.2 g/l; $K_2HPO_4 \cdot 3H_2O$, 0.5 g/l; pH 7.0) supplemented with 100 μg/ml streptomycin and 50 μg/ml kanamycin. Bacteria were collected by centrifugation at 5500 × *g* for 20 min and then resuspended in infiltration medium (5.0% sucrose and 0.05% Silwet L-77) to a final OD_{600} of approximately 0.80.

Transformation was carried out using the floral dip method as described previously (Clough and Bent, 1998). The above-ground tissues of the flowering plants were inverted into the bacterium suspension for 3–5 s with gentle agitation and kept in a dark room with 100% of humidity for 24 h. Plants were returned to the growth chamber and grown for a further 3–5 weeks to set seeds.

Seeds were surface sterilized by treatment with 95% ethanol for 60 s, then with 50% bleach (2.625% sodium hypochlorite) containing 0.05% Tween 20 for 5 min, followed by four washings with sterile water. The sterilized seeds were suspended in 0.1% agarose and cold-treated for 2 days at 4 °C. The seeds were then plated on 1/2× MS, 0.8% agar, 50 μg/ml kanamycin at a density of 2000 seeds per petri dish (9

cm in diameter) and allowed to grow for 7 days at 24 °C with 20 h of light (120 μE m^{-2} $^{-1}$). Putative transformants with dark-green leaves and well-established roots in the selective medium were transferred to soil and grown in the growth chamber to set seeds.

2.8. Assay for GUS activity

More than ten independent transgenic lines for each promoter deletion constructs and the empty vector were used for GUS analysis. Three-week-old seedlings of transformant *A. thaliana* and wild-type were treated by spraying with 1 mM SA, 0.3 mM BTH, 0.3 mM INA or water. Samples were collected from at least ten seedlings 2 days after treatment and frozen immediately in liquid nitrogen. Leaf tissues were ground in the GUS extraction buffer (50 mM sodium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) and the clear supernatants were used for GUS assays. GUS activity was measured fluorometrically using 4-methylumbelliferyl glucuronide as substrate according to Jefferson's protocol (Jefferson, 1987). Measurement of fluorescence was performed with THO100 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA) at 460 nm using 4-methylumbelliferone (MU) as standard. Protein concentrations in the extracts were determined by Bradford method (Bio-Rad Laboratories) according to the manufacturer's instructions. The specific activity was expressed as pmol MU min^{-1} (mg protein)$^{-1}$.

3. Results

3.1. Expression of the tobacco Sar8.2b gene is SA-dependent

The *Sar8.2* gene family contains at least 12 members and each member shows a different pattern of expression upon different stimuli including treatment with SAR inducers, wounding and touch (Moraes and Goodman, unpublished). Accumulation of *Sar8.2* mRNAs was found to be SA-dependent upon TMV or SA treatment; among these members, *Sar8.2b* and *Sar8.2l* have been demonstrated to correlate with the SAR-related *Sar8.2* mRNA accumulation. Therefore, we first analyzed the expression kinetics of the *Sar8.2b* gene during SAR induced by SA and the expression pattern in wild-type and *NahG* transgenic plants after treatment with different SAR inducers.

Some members of the *Sar8.2* gene family were expressed constitutively in roots, stems and leaves of tobacco plants at levels that varied significantly between experiments (Ward et al., 1991). Notwithstanding its relatively high constitutive expression background in some experiments, however, we found that expression of the *Sar8.2b* gene consistently increased after treatments with the SAR inducers SA, INA or BTH (Fig. 1B). In the time-course study, no significant expression of the *Sar8.2b* and *PR-1a* genes was detected during the experimental period (Fig. 1A). Expression of the *Sar8.2b* gene was detected as early as 12 h after SA treatment in the treated leaves and was maximal 36 h after treatment (Fig. 1A). The kinetics of the *Sar8.2b* gene expression upon SA treatment was similar to that of *PR-1a* (Fig. 1A).

We then analyzed the expression of the *Sar8.2b* gene in wild-type and in *NahG* transgenic tobacco plants after treatments with different SAR inducers. In wild-type plants, treatments with SA, BTH or INA strongly increased expression of the *Sar8.2b* gene (Fig. 1B). The expression level in *NahG* transgenic plants was increased significantly after treatment with BTH or INA; however, no expression was detected after SA treatment. This result indicates that expression of the *Sar8.2b* gene is SA-dependent.

3.2. Cloning of the Sar8.2b gene promoter

To better understand the mechanism by which expression of the *Sar8.2b* gene is regulated during SAR induction, we cloned and analyzed the promoter region of the gene. We started with a phage DNA from a tobacco genomic clone, designated 183, which contains the *Sar8.2b* gene (Moraes and Goodman, unpublished; GenBank accession no. U64816). We amplified the upstream region of the gene by

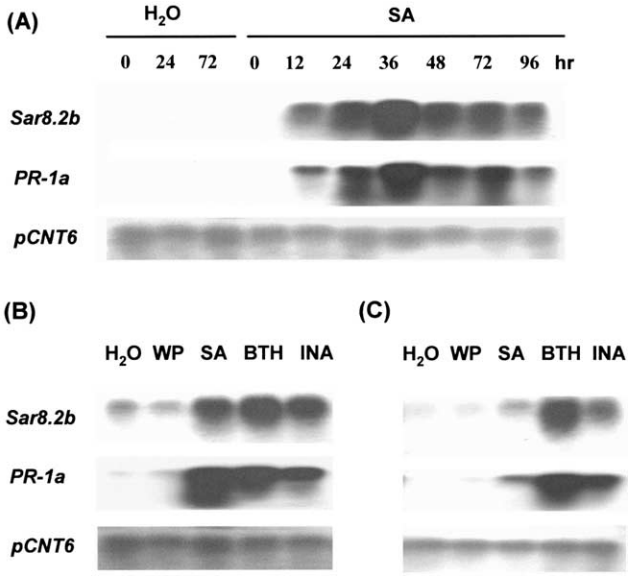


Fig. 1. Expression of the *Sar8.2b* gene upon treatment with chemical inducers. (A) Expression kinetics of the *Sar8.2b* gene in tobacco plants after treatment with salicylic acid (SA). (B,C) Expression of the *Sar8.2b* gene in wild-type and the *NahG* transgenic tobacco plants 3 days after treatment with SA, benzothiadiazole (BTH), dichloroisonicotinic acid (INA), wettable powder (WP) or water. Four-week-old wild-type and *NahG* transgenic tobacco (Xanthi nc) plants were treated by spraying with solutions of 2 mM SA, 0.3 mM BTH, 0.3 mM INA, WP and water onto one fully expanded leaf and the leaf samples were collected at each time point as indicated. Ten micrograms of total RNA were fractionated on formaldehyde–agarose gel, transferred onto nylon membrane and then hybridized with DIG-labeled *Sar8.2b* and *PR-1a* probes as described in Section 2. The tobacco gene *pCNT6* was used for evaluation of equal loading.

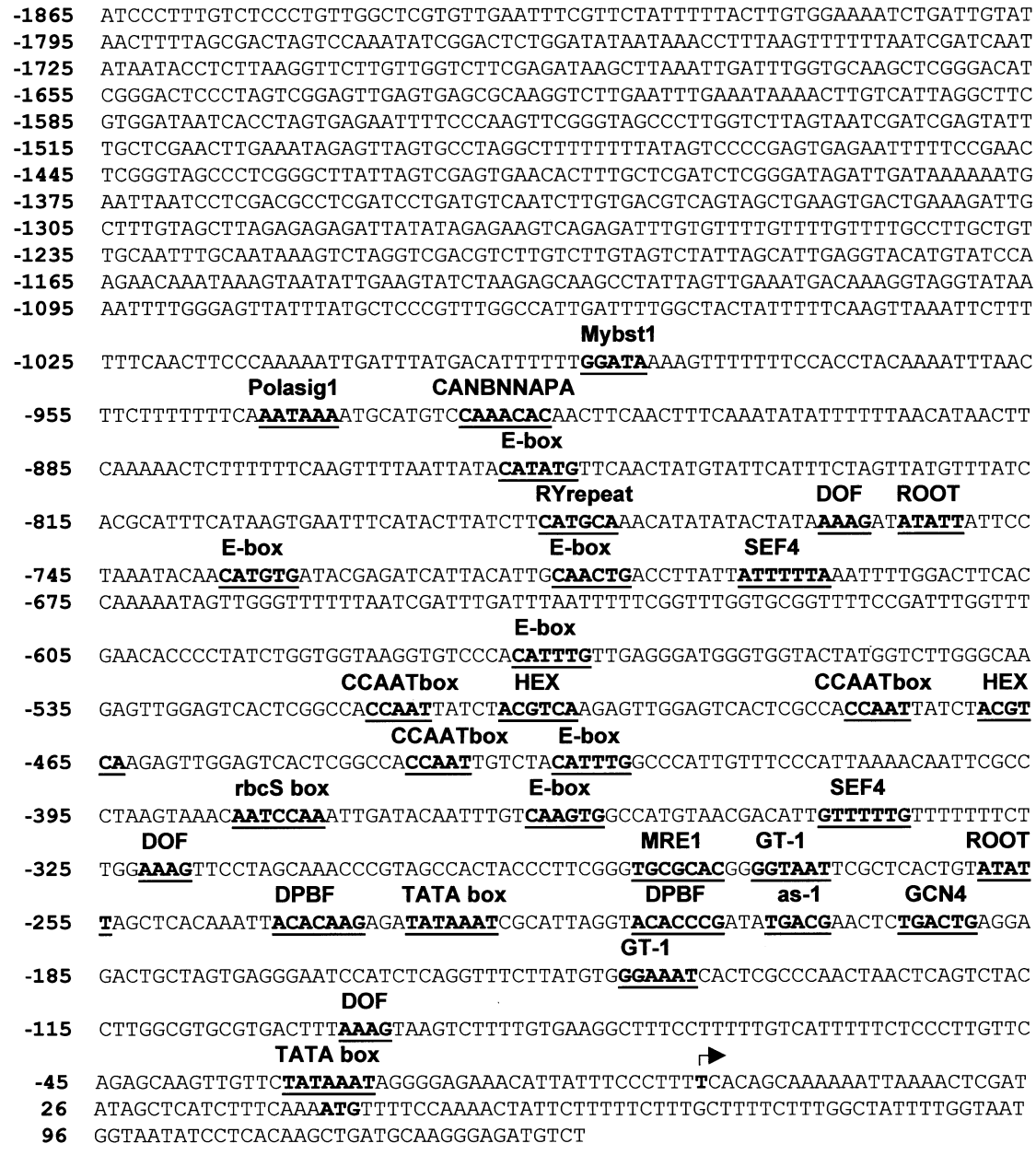


Fig. 2. Nucleotide sequence of the tobacco *Sar8.2b* gene promoter and putative *cis*-acting elements. The start codon (ATG) is in bold and the transcriptional start site is marked by an arrow. The putative *cis*-acting elements are underlined and the names are given above the elements.

PCR using a vector primer and a *Sar8.2b* gene-specific primer. The sequences of 128 and 303 bp at the 3′ end of this fragment are identical to the sequences of the *Sar8.2b* cDNA (GenBank M97359) and its gene (GenBank U64816), respectively. Therefore, the cloned fragment is most likely to be the promoter region of the *Sar8.2 b* gene (Fig. 2).

5′-RACE was used to map the transcriptional start site of the *Sar8.2b* gene. A ~500 bp fragment was amplified in 5′-RACE using two *Sar8.2b* gene-specific nested primers (data not shown). After sequencing and alignment with the cloned *Sar8.2b* gene promoter region, the transcriptional start site was mapped to the 44th nucleotide (a T in a stretch of AT-rich sequence) upstream of the ATG codon (Fig. 2).

The cloned *Sar8.2b* gene promoter region is 1865 bp in length (Fig. 2). Two TATA boxes (TATAAAT) are recognized at −31 to −25 and −232 to −226, respectively. Searches for putative *cis*-acting elements in the promoter region were performed using PLACE (a database for PLAnt Cis-acting Elements at http://www.dna.affrc.go.jp). Sixteen types of putative *cis*-acting elements were found in the promoter region (Fig. 2). Notably, we found one *as-1* (−205 to −201), one Mybst1 (−989 to −985), two GT-1 binding sequences (−146 to −141; −276 to −271), and three Dof binding motifs (−97 to −94; −322 to −318; −761 to −758) elements. The *as-1* or *as-1*-like elements and GT-1 elements were previously found in tobacco *PR-1a*

and Arabidopsis *PR-1* gene promoters (Strompen et al., 1998; Lebel et al., 1998; Buchel et al., 1996) and shown to bind with transcription factors that are SA-responsive (Qin et al., 1994; Strompen et al., 1998; Buchel et al., 1996). SA-inducible Myb and Dof proteins were also found in tobacco and Arabidopsis (Yang and Klessig, 1996; Kang and Singh, 2000).

3.3. Identification of promoter activity in the cloned promoter region of the Sar8.2b gene

To determine the minimum sequence of the cloned promoter region of the *Sar8.2b* gene required for promoter activity, we constructed nine deletion mutations from the 5′ end of the cloned *Sar8.2b* gene promoter region and fused to GUS reporter gene (Fig. 3). Deletion mutations C, E, G and H were constructed by digesting the plasmid containing the cloned promote region with *Bam*HI and one other restriction enzyme and contain 927, 647, 435 and 351 bp of the promoter region, respectively. Deletion mutations B, D, F, I, and K were created by digesting the plasmid with Exonuclease III and contain 1140, 728, 564, 197 and 102 bp of the promoter region, respectively.

We performed transformation of *A. thaliana* ecotype Columbia with the 5′ deletion constructs of the tobacco *Sar8.2b* gene promoter region (Fig. 3) as well as the empty vector using the floral dip method (Clough and Bent, 1998). Stable transformants were screened and used for GUS analysis. We first tested whether treatments with SA, BTH and INA, three chemical inducers that induce expression of the *Sar8.2b* gene (Fig. 1B), led to expression of GUS driven by the *Sar8.2b* gene promoter in *A. thaliana*. As shown in Fig. 4, GUS stain was observed only in leaves of the transgenic *A. thaliana* seedlings after treatments with SA, BTH and INA. Often, GUS stain was seen in the young and fully expanded leaves; but in some cases, it was also seen in older leaves. In the wild-type and empty vector-transformed *A. thaliana* seedlings, no visible GUS stain was detected both in water control and SA treatment. Likewise, no GUS stain was detected in leaves of transgenic *A. thaliana* seedlings after treatment with water, suggesting that expression of GUS gene driven by the *Sar8.2b* gene promoter was only activated upon induction by SAR inducers (the right three plants in the upper row of Fig. 4). This result shows that the tobacco *Sar8.2b* gene promoter can function in *A. thaliana* as it does in tobacco.

We then analyzed the GUS activity in transformed *A. thaliana* after treatment with SA to determine the minimal sequence required for promoter activity. Only the kanamycin-resistant seedlings grown from T_1 seeds from at least ten independent transgenic lines were used for GUS activity analysis. Leaf samples were collected 2 days after treatment with SA or water and the protein extracts were subjected to analysis of GUS activity. Induction of GUS activity was detected in transgenic *A. thaliana* seedlings transformed with the constructs containing fragments of −197 bp or longer in the cloned promoter region after SA treatment (Fig. 5). Fragments of 927 bp or longer of the cloned promoter region (constructs A, B and C) showed similar induction in GUS activity by SA, giving 26–31-fold increase in GUS activity after SA treatment. This indicated that the 927 bp fragment of the *Sar8.2b* gene promoter confers full promoter activity. Deletion of the sequence between −728 and −928 bp led to a significant decrease in GUS activity induced by SA (construct D); deletion between 728 and 564 bp slightly reduced the induction of GUS (construct F). No difference in induction of GUS activity by deletions between −564 and −435 bp was detected (constructs F and G), showing ~15-fold induction of GUS activity by SA treatment. Further deletion between −435 and −351 bp also slightly reduced SA-inducible GUS activity (construct H). Deletion between −351 and −197 bp significantly

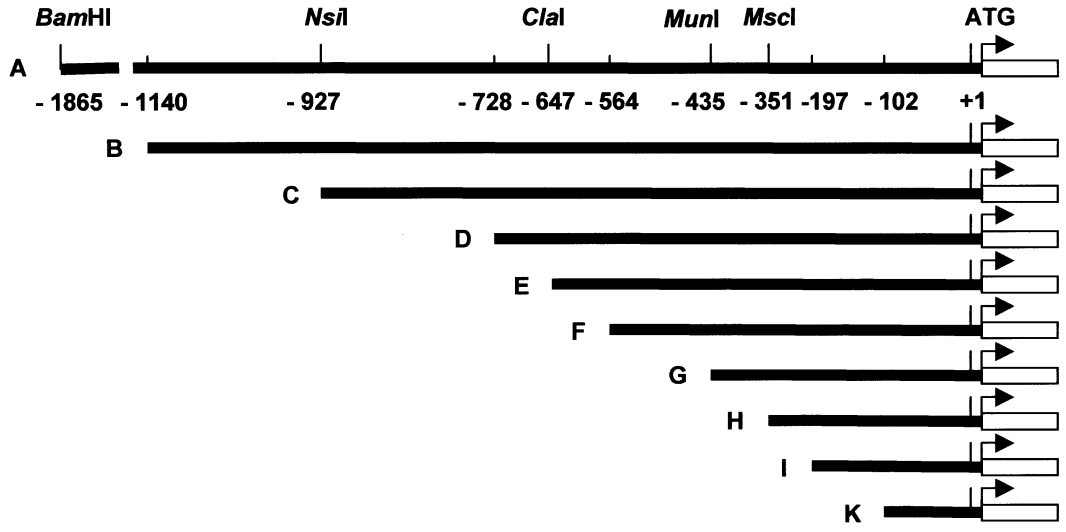


Fig. 3. Diagrammatic representation of the progressive deletion constructs of the tobacco *Sar8.2b* gene promoter fused to *gus* reporter gene.

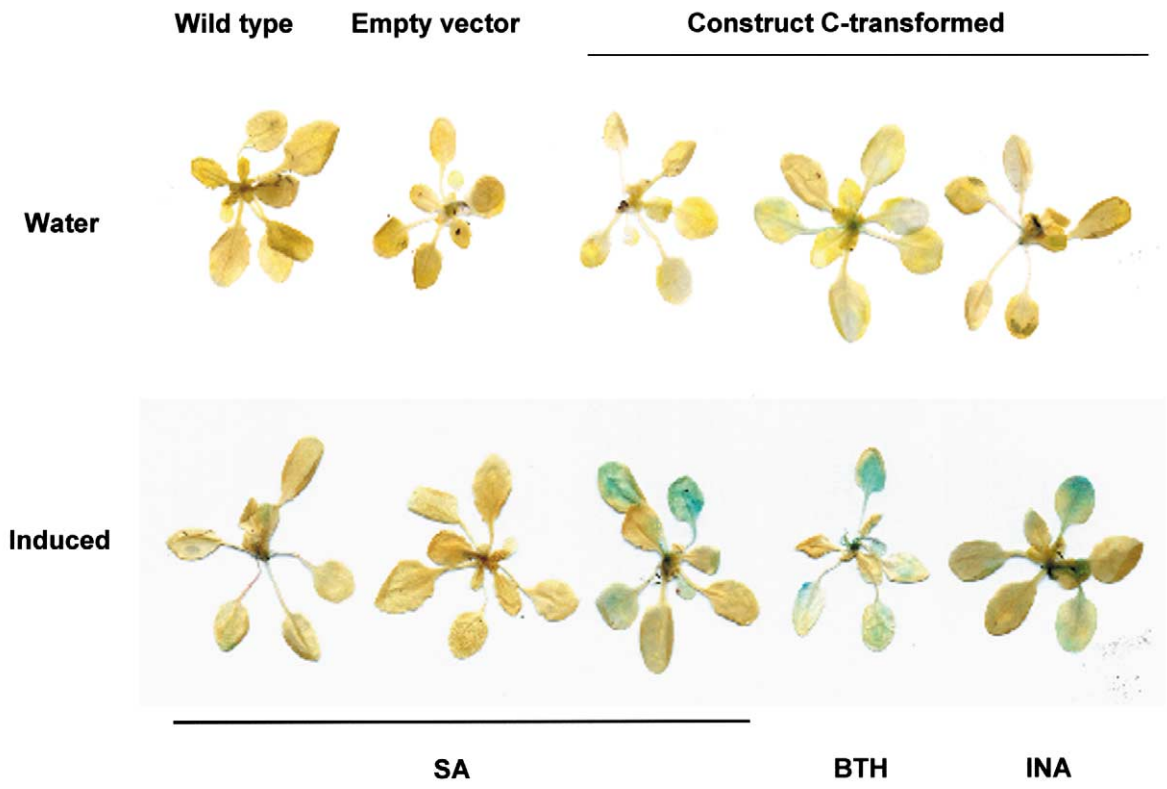
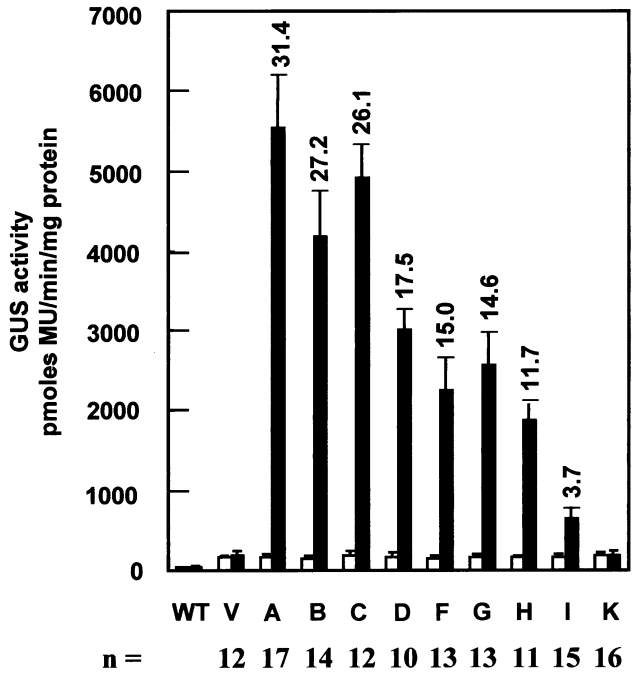


Fig. 4. GUS staining in stable transformants and wild-type of *Arabidopsis thaliana* seedlings after treatment with SAR inducers. Three-week-old *A. thaliana* seedlings were treated by foliar spraying with 1 mM salicylic acid (SA), 0.3 mM benzothiadiazole (BTH), 0.3 mM dichloroisonicotinic acid (INA) or water as indicated and collected for GUS staining 2 days after treatment.

decreased the induction of GUS activity by SA; however, −197 bp fragment of the promoter region also resulted in ~4-fold induction of GUS by SA treatment (construct I). No SA-induced GUS activity was seen with the shortest construct tested (construct K).



4. Discussion

In this study, we demonstrated that expression of the *Sar8.2b* gene, a member of the *Sar8.2* gene family (Alexander et al., 1992), is activated in a SA-dependent manner and we cloned and identified the putative promoter region of the gene. The kinetics of *Sar8.2b* gene expression in response to SA treatment was found to be similar to that of *PR-1a*. However, expression of the *Sar8.2* gene members was activated much more rapidly in tobacco treated with SA by injection (Guo et al., 2000). This difference may result from the difference in uptake of SA between the two methods used for treatment (Xie et al., 1998). BTH and INA were also shown to activate expression of the *Sar8.2b* gene, which is consistent with results with other members of the gene family that are induced by BTH and INA both in

Fig. 5. Deletion analysis of the tobacco *Sar8.2b* gene promoter in transformants of *Arabidopsis thaliana*. GUS activity for the deletion constructs of the tobacco *Sar8.2b* gene promoter after treatment with water (open bars) or with salicylic acid (SA, closed bars). WT, wild type; V, empty vector only; A to K, deletion constructs of the tobacco *Sar8.2b* gene promoter A to K (Fig. 3). The average of GUS activity and the standard deviations of experimental data from independent assays of extracts from different transgenic *A. thaliana* lines are shown. The numbers above the bars indicate the fold increases of GUS activity after SA treatment vs. activity without SA treatment. The number of independent transgenic *A. thaliana* lines used for each construct is indicated (n).

detached leaf disc or intact plant leaves (Guo et al., 2000; Wendenhenne et al., 1998). In our study, SA did not induce expression of the *Sar8.2b* gene but BTH and INA both activated its expression in *NahG* tobacco plants. This is different from the observation that TMV infection induced expression of other member(s) of the *Sar8.2* gene family in *NahG* tobacco plants, which indicated that expression of these members is SA-independent (Guo et al., 2000). The contrast between these results may imply that expression of the *Sar8.2* gene family members requires different signaling pathways, an interpretation consistent with our previous observation that different members of the *Sar8.2* gene family show different expression patterns upon different stimuli (Moraes and Goodman, unpublished). The ethylene action inhibitor silver thiosulfate significantly reduced the expression of *Sar8.2* genes induced by TMV infection, implying that expression of some member of the *Sar8.2* gene family may require ethylene (Guo et al., 2000).

The *Sar8.2* gene family was originally identified in *Nicotiana tabacum* (Alexander et al., 1992). It was later found that other *Nicotiana* species as well as potato and tomato might also contain *Sar8*.2 orthologous genes (Moraes and Goodman, unpublished). However, no *Sar8.2* orthologous gene was detected in *A. thaliana* by RT–PCR or Southern blot analysis (Moraes and Goodman, unpublished). We transformed *A. thaliana* with different deletion constructs of the tobacco *Sar8.2b* gene promoter region and found that expression of the GUS gene driven by the *Sar8.2b* gene promoter region was also induced by treatment with SA, BTH or INA (Fig. 4). This result suggests that the *cis*-acting elements of the tobacco *Sar8.2b* gene promoter can be recognized and bound by the transcription factors in *A. thaliana* and thus regulate GUS gene expression. Recently, Kirsch et al. (2001) reported that a *cis*-acting element identified from *CMPG1* in *Petroselinum crispum* could drive expression of the GUS reporter gene in transgenic *A. thaliana* plants when treated with flagellin peptide elicitors or infected with pathogens. Because transformed *Arabidopsis* plants are easier to obtain, this method may enable researchers to identify the activity of promoters from which the host plants are difficult to be transformed.

In our study, we found that the 927 bp upstream sequence of the *Sar8.2b* gene promoter is sufficient for full promoter activity. Further deletion of this region reduced the promoter activity in the transgenic *A. thaliana* plants upon SA induction. The results suggest that this 927 bp upstream sequence in the *Sar8.2b* gene promoter may be the minimal promoter length for promoter function. This is similar to previous results found in the *PR-1a* gene promoters of tobacco and *A. thaliana*, where the ~900 bp and 815 bp of the upstream sequences are the minimal promoter lengths for high level induction of the reporter gene in response to all stimuli (Uknes et al., 1993; Grüner and Pfitzner, 1994; van de Rhee et al., 1990; Lebel et al., 1998). In the tobacco *PR-1a* gene promoter, 0.3 kb of upstream sequence is sufficient for regulated expression of the GUS reporter gene induced by SA (Ohshima et al., 1990), and two independent nuclear protein binding sites that might be involved in negative regulation of the *PR-1a* gene expression were also identified between −184 and −172, and between −68 and −51 within this region (Hagiwara et al., 1993). We found that the −197 bp upstream region of the *Sar8.2b* gene promoter region yielded a low level of SA-inducible GUS activity in stable transgenic *A. thaliana* plants after SA induction, indicating that this upstream region may be necessary for regulating basal induction of the *Sar8.2b* gene expression.

Several well-identified *cis*-acting elements that are previously shown to be involved in regulation of SAR gene expression were found in the cloned promoter region of the tobacco *Sar8.2b* gene (Fig. 2). The *as-1*, GT-1, Dof and mybst1 elements have been previously identified in promoters of some SAR genes (Strompen et al., 1998; Lebel et al., 1998; Buchel et al., 1996). The transcription factors capable of binding to these *cis*-acting elements have also been shown to respond rapidly to SA as well as other signals such as auxin, jasmonate and H_2O_2 (Buchel et al., 1996, 1999; Jupin and Chua, 1996; Stange et al., 1997; Ulmasov et al., 1994; Xiang et al., 1996; Zhang and Singh, 1994; Niggeweg et al., 2000; Yang and Klessig, 1996; Kang and Singh, 2000). However, whether these putative *cis*-acting elements play a role, and if so, how they function, in regulation of the tobacco *Sar8.2b* gene expression after SA induction, require further study.

In our deletion analysis of the tobacco *Sar8.2b* gene promoter, deletion of sequence between −728 and −928 bp or between −351 and −197 bp of the promoter region resulted in a significant reduction in GUS activity induced by SA treatment, implying that *cis*-acting elements required for high-level SA-inducible expression of the *Sar8.2b* gene may exist within these regions. An *as-1* element and a Dof binding sequence, which were shown to be SA-responsive (Jupin and Chua, 1996; Stange et al., 1997; Kang and Singh, 2000; Xiang et al., 1996; Niggeweg et al., 2000), were found within −197 to −351 bp and −728 to −928 bp of the promoter region, respectively. Surprisingly, we found that the −197 bp fragment of the promoter conferred SA-inducible GUS activity in stable transformants of *A. thaliana* after SA treatment (Fig. 5). This suggests that the *as-1* element, located at −205 to −201, may only be required for high-level induction by SA but not necessary for the *Sar8.2b* gene expression. This is in agreement with the observation that deletion and mutation in the *as-1* element sequence of the tobacco *PR-1a* gene promoter significantly reduced but did not abolish the ability of SA to activate its expression in tobacco leaf disc (Strompen et al., 1998). These results imply that *as-1* element and Dof binding sequence in the tobacco *Sar8.2b* gene promoter region may play an important role in regulation of its expression during SA induction. However, further work is required to test whether these elements are responsible for the high-level induction by SA of the *Sar8.2b* gene expression.

The role of the *Sar8.2* gene family in disease resistance

has been demonstrated not only by the tight correlation of its expression with SAR but also by the observation that transgenic tobacco plants constitutively expressing a member of this family showed enhanced disease resistance (Alexander et al., 1993; Guo et al., 2000; Chen and Goodman, unpublished). However, the signaling pathway(s) leading to induction of gene expression of this family and the mechanisms by which their expression is regulated upon induction by SAR inducers remain unclear. Our cloning and characterization of the *Sar8.2b* gene promoter will facilitate studies on these aspects. Further work may include characterization of *cis*-acting elements conferring high-level induction of gene expression by SA and identification of the *trans*-acting factors that can bind to the *cis*-acting elements and are SA-responsive.

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