Tobacco Transcription Factor TGA2.2 Is the Main Component of as-1-binding Factor ASF-1 and Is Involved in Salicylic Acid- and Auxin-inducible Expression of as-1-containing Target Promoters*

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In higher plants, activating sequence-1 (as-1) of the cauliflower mosaic virus 35 S promoter mediates both salicylic acid (SA)- and auxin-inducible transcriptional activation. Originally found in promoters of several viral and bacterial plant pathogens, as-1-like elements are also functional elements of plant promoters activated in the course of a defense response upon pathogen attack. Nuclear as-1-binding factor (ASF-1) and cellular salicylic acid response protein (SARP) bind specifically to as-1. Four different Tobacco bZIP transcription factors (TGA1a, PG13, TGA2.1, and TGA2.2) are potential components of either ASF-1 or SARP. Here we show that ASF-1 and SARP are very similar in their composition. TGA2.2 is a major component of either complex, as shown by supershift analysis and Western blot analysis of DNA affinity-purified SARP. Minor amounts of a protein immunologically related to TGA2.1 were detected, whereas TGA1a was not detectable. Overexpression of either TGA2.2 or a dominant negative TGA2.2 mutant affected both SA and auxin (2,4-D) inducibility of various target promoters encoding as-1-like elements, albeit to different extents. This indicates that TGA2.2 is a component of the enhancosome assembling on these target promoters, both under elevated SA and 2,4-D concentrations. However, the effect of altered TGA2.2 levels on gene expression was more pronounced upon SA treatment than upon 2,4-D treatment.

Plants activate a spectrum of defense genes upon pathogen attack. Recognition of the pathogen is followed by the activation of different signal transduction chains, which are functionally connected to a large number of transcription factors regulating the activity of defense gene promoters (1). One of these defense responses, known as systemic acquired resistance (SAR),1 confers immunity against a broad spectrum of pathogenic upon local infection with a necrogenic pathogen (2). SAR is associated with the co-ordinate induction of a set of host genes presumably involved in plant defense mechanisms such as glucanases, chitinases, and pathogenesis-related (PR) proteins (3, 4). Salicylic acid (10 μM SA) is both necessary and sufficient to induce this set of genes (3, 5) and to confer SAR. Activating sequence-1 (as-1) is a functionally important element of a subgroup of SA-inducible defense genes (e.g. Nt103, also referred to as GNT35 (Ref. 6), and PR-1a (Ref. 7)). Although elevated levels of SA (1 mM) are required to induce the isolated element (8), its regulation is likely to affect the expression of several SAR genes. This is corroborated by the finding that as-1-binding bZIP transcription factors of the TGA family interact with ankyrin-rich repeat protein NPR1 (nonexpressor of PR-1), which is essential for SA-inducible expression of PR-1 in Arabidopsis thaliana (9–11). The isolated element also responds to elevated levels of auxin (50 μM 2,4-D; Ref. 12), indicating its functional connection to other signal transduction pathways independent from the SAR.

as-1-like elements are characterized by two imperfect TGACGTCA motifs spaced by 4 bp (13). Using electrophoretic mobility shift assays (EMSAs), a nuclear protein complex called ASF-1 was identified (13). ASF-1 is most likely involved in SA induction of the as-1 element, as oligonucleotides encoding two perfect TGACGTCA motifs are recognized with higher affinity by ASF-1 and confer higher SA inducibility of transcription (8). An as-1 binding activity identified in leaf cell extracts has been called salicylic acid response protein (SARP; Ref. 14). Its main component is a 40-kDa protein that is immunologically related to bZIP transcription factor TGA1a. Both SARP activity and ASF-1 activity increase upon SA treatment of leaves (14, 15). Biochemical evidence supports the hypothesis that, in the absence of SA, binding of SARP to as-1 is inhibited by a not yet identified protein (SAI). Knowledge of the molecular composition of ASF-1 or SARP will be a crucial step toward the identification of SAI.

TGA1a was the first of the four so far characterized members of the tobacco as-1-binding family of bZIP factors to be isolated (16). A related tobacco clone called PG13 was subsequently reported (17). In A. thaliana, cDNA clones coding for seven TGA-like factors (TGA1 to TGA6 and PERIANTHIA; Refs. 18–23) have been isolated and grouped into three distinct classes based on sequence similarities. The bZIP domains of these factors are highly conserved, conferring similar DNA binding specificities and heterodimerization properties. Tobacco TGA1a and PG13 are grouped into class I. Using class II A. thaliana TGA5 cDNA as a probe, we have isolated cDNAs phosphate-buffered saline with Tween 20; bp, base pair(s); PCR, polymerase chain reaction.
coding for Nicotiana tabacum TGA2.1 (24) and N. tabacum TGA2.2 (25). N. tabacum TGA2.2 is 79% identical to A. thaliana TGA2 at the amino acid level. TGA2.1 is 76% identical to A. thaliana TGA2, but is characterized by an additional 128-amino acid N-terminal domain.

At present, information on the in vivo function of individual TGA factors is limited. In Arabidopsis, the perianthia mutant is characterized by five instead of four petals, but the target genes of PERIANTHIA are not yet known (23). Injection of recombinant TGA1a into tobacco leaf cells led to transcriptional activation from the as-1 element (26), indicating that the amount of TGA1a can lead to constitutive promoter activation. TGA1a is predominantly expressed in roots (16). This observation has been taken as evidence that TGA1a is responsible for root specific expression from the element. In addition, increased TGA1a levels lead to increased activation of the as-1 element upon 2,4-D treatment, as revealed by transient expression analysis using protoplasts from tobacco cell line BY-2 (27).

In this study, we elucidate the composition of ASF-1 and SAR and thus define two members of the TGA family (TGA2.1 and TGA2.2) as candidates for being involved in regulating the as-1 element in leaves. Biochemical analysis identified the TGA2.2 homodimer as the main component of these complexes. Functional analysis of transgenic plants expressing either TGA2.2 and or a dominant negative TGA2.2 mutant supports its involvement in gene expression in leaves as expression of genes containing the as-1 element within their regulatory sequences was altered both in response to SA and in response to 2,4-D.

EXPERIMENTAL PROCEDURES

In Vitro Transcription/Translation—The coding regions of TGA2.1, TGA2.2 and TGA1a were cloned into plBluescript SK under the control of the T7 RNA polymerase promoter (25). RNAs were synthesized using the T7 transcription kit (MBI) with 1 µg of plasmid DNA. TGA proteins were obtained using the rabbit reticulocyte lysate system (Promega). 0.5 µg of RNA were included in each reaction. To control the efficient synthesis of the different factors, products were radiolabeled by [35S]methionine incorporation and separated on a 10% SDS-PAGE (28).

The gel was incubated in 50% methanol, 10% glacial acetic acid for 30 min and in 7% acetic acid, 7% methanol, 1% glycerol for 5 min before drying it on a gel dryer. The gel was exposed to a Bioimager screen overnight.

EMSAs—For EMSAs using as-1 as a probe, a 77-bp HindIII/EcoRI fragment was cut out from the pUC18-as1 vector. This vector was constructed by cloning the following oligonucleotide into the XbaI site of the multiple cloning site of pUC18: HindIII-XbaI-TGACGTAAaggTAGCCGA-C-XbaI-EcoRI.

In addition, the 274-bp 5xas-1 PCR fragment described below (see “Affinity Purification of SARP”) was amplified with nonbiotinylated primers and cloned into the vector pUC57-T (MBI Fermentas). For radio-labeling, a 250-bp fragment was cut out using the internal EcoRI site of the PCR fragment and the XbaI site of the vector.

As a box probe, a 55-bp XbaI/EcoRI fragment was cut out of the pUC18-G-box vector. This vector was constructed by cloning a 28-bp BamHII/BgII fragment containing the G-box element, which was obtained from the plasmid pBECK box II (kindly provided by Dr. B. Weisshaar, Max Planck Institute for Plant Breeding, Cologne, Germany), into the BamHI site of the pUC18 vector. XbaI-GATCCCTATTACC-ACTGCCCATCGGAGATCCCGGTACCGAGCTC-EcoRI (the G-box motif is written in bold letters).

Functional analysis of TGA2.2 was performed with synthetic peptide encoding the basic domain (RRLAQREARSKRRPKK) was coupled to ovalbumin (Pierce) and used for rabbit immunization. The antibody was purified against the peptide coupled to CNBr-activated Sepharose according to a procedure recommended by Amersham Pharmacia Biotech. The antibodies directed against TGA2.1 and TGA2.2 were affinity-purified using protein extracts from recombinant Helicobacter coli strain as described (26).

Expression of Recombinant TGA Factors in E. coli—The cDNAs encoding either TGA2.1 or TGA2.2 were excised from the respective pSK vectors (25) as KpnI/BamHI fragments and ligated to the protein expression vector pET3a and DNA polymerase promoter. The pET3a vector is a derivative of the vector pET3b with further restriction enzyme sites (KpnI, SnaBI, and HindIII) added to the multiple cloning site downstream of the ATG start codon. This cloning strategy added three amino acids to the N termini of TGA2.1 and TGA2.2. These constructs were transformed into E. coli BL21(DE) competent cells. To express TGA1a, a ϕ vector containing the cDNA of TGA1a (7) was used; the vector was kindly provided by Dr. U. Pfitzner (University of Hohenheim, Hohenheim, Germany). 0.4 ml of an overnight culture was added to 20 ml of medium and incubated at 37 °C until A600 = 0.6–0.8 was reached. The expression of the encoded protein was induced for 3 h with 0.6 mM isopropyl-β-D-thiogalactoside. The pellet of the cells was resuspended in 10 volumes of denaturation buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5). Western Blot Analysis—Tobacco and bacterial proteins were first separated on a 10% SDS-PAGE (28) and then transferred onto a polyvinylidene difluoride membrane (Millipore) as described (31). The membrane was blocked in PBS-T (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 4 mM Na2HPO4, 0.05% Tween 20) containing 5% nonfat dried milk overnight at 4 °C by shaking. It was washed once for 5 min in PBS-T containing 1% nonfat dried milk and then incubated with the respective affinity-purified antibodies diluted in 1% nonfat dried milk/PBS-T for 2 h at room temperature. The membranes were washed in 1% nonfat dried milk/PBS-T four times for 5 min. They were incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody at a dilution of 1:5000 in PBS-T for 1.5 h at room temperature, washed four times for 5 min in PBS-T and twice for 5 min in PBS. Detection was carried out with a chemiluminescence reaction using the ECL detection kit (Amersham Pharmacia Biotech) according to manufacturer’s protocols. The membrane was finally exposed to autoradiography film (Cronex T10, Eastman Kodak Co.). Before incubation of a processed filter with a second primary antibody, it was washed once with PBS and then incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 0.1 M Tris-HCl, pH 6.8) for 60 min at 50 °C by shaking. Washing was performed twice in PBS-T.

Plant Material and Treatments—Tobacco plants (N. tabacum cv. Samsun NN) were grown under a 16-h light (22 °C)/8-h (19 °C) regime. Fully expanded, young leaves from tobacco plants were taken either for protein or RNA extraction. For induction experiments, days of these leaves were floated on 1% sucrose and L-glutathione acid (SA) or 2,4-D, or 0.6–0.8 M 2,6-dichloro-p-phenylbenzoic acid (HBA) in 10 mM phosphate buffer, pH 6.8, or on 50 µM 2,4-dichlorophenolacetic acid (2,4D) or 50 µM 2,6-dichlorophenoxyacetic acid (2,6D) in 50 mM phosphate buffer, pH 5.7. These treatments were performed in a climate chamber under a 16-h light (24 °C)/8-h (22 °C) regime.

Preparation of Protein Extracts from Tobacco Leaves—Preparation of nuclei was done as described previously (29). Nuclei were not purified using Percoll, but directly used for nuclear protein extraction as described previously (32). Cellular extracts were prepared according to Jupe and Chua (14). Instead of using Centricon tubes, conventional dialysis was used to desalt the extract. This alteration did not change the binding characteristics of SARP.

Cell Culture and Preparation of Cellular Extracts from BY 2 Cells—Bright Yellow-2 (BY-2) tobacco (N. tabacum cv. N. tabacum cv) cell suspension cultures were grown in a modified Murashige and Skoog medium with 3% sucrose, 0.9 g/liter myo-inositol, 0.9 mg/liter thiamine, and 0.22 mg/liter 2,4-D at 26 °C in the dark on a rotary shaker. Every week the cells were subcultured by transferring 3 ml of the culture to 27 ml of fresh medium. Cells were harvested 4 days after subculture by suctioning off the medium, frozen in liquid nitrogen, and stored at −80 °C. After freeze drying the frozen cells in nitrogen, the freeze-dried powder was used to prepare cellular extracts as described above for cellular extracts from tobacco leaves. Typically, 2 ml of cellular extracts containing about 5 mg of protein/ml were obtained from 2 g of BY-2 cells.

Affinity Purification of SARP: Preparation of DNA Affinity Beads—A 274-bp PCR fragment containing five as-1 elements (5xas-1) was amplified with a 5′ biotinylated 27-bp upstream primer and a 23-bp down-
Salicylic Acid- and 2,4-D-responsive bZIP Factor

TGA2.2 Is the Main Component of ASF-1 and SARP—The presence of multiple TGA factor encoding genes in tobacco raised the following question: which members of this family constitute as-1-binding complexes present in nuclear extracts (ASF-1) and whole cell extracts (SARP)? Supershift analysis with antibodies designed to specifically recognize individual TGA factors provides a highly sensitive assay for the analysis of the composition of DNA-binding complexes (43). Antibodies directed against the N-terminal domains of TGA1a, TGA2.1, and TGA2.2 were generated and shown to be highly specific in supershift analysis using in vitro translated proteins (25). A 77-bp-long DNA fragment encoding as-1 was used as a probe for supershift analysis of either ASF-1 or SARP. ASF-1 and SARP had similar mobilities indicating that both complexes might represent the same activity (Fig. 1, a and b). Strong supershifting was only observed upon addition of the anti-TGA2.2 serum either to nuclear extracts or to whole cell extracts. The amount of radioactive probe found in the supershifted complexes corresponded to the amount lost in the ASF-1/ as-1 or SARP/ as-1 complex. Supershifted complexes were also obtained with anti-TGA2.2 serum, although to a lesser extent than with the anti-TGA2.2 serum. Quantification of three independent experiments revealed that ~80% of ASF or SARP were supershifted with the anti-TGA2.2 serum, whereas the anti-TGA2.1 serum recognized 10% of ASF-1 and 20% of SARP. We failed to detect any TGA1a in either complex.

Next, we approached the question of whether a (so far) unknown TGA factor might be present in SARP. To be able to characterize such a protein, an antibody directed against the conserved basic DNA-binding domain of TGA factors was performed. This antibody also recognizes other plant bZIP factors (e.g. G-box binding factors (GBFs), Ref. 44), cellular extracts were enriched for TGA factors before subjecting them to Western blot analysis. Expression of TGA2.2 and TGA2.2trd in Tobacco—To introduce the mutations in the basic domain of TGA2.2, a cDNA fragment encoding the N terminus and basic domain of TGA2.2 was amplified by PCR. The lower primer (CCG ACC TCT CAT ATG CCT TTT TCC GGC GTG TGT CAC G) introducing a HindIII site was complementary to the basic DNA-binding domain containing nucleotide substitutions to change the sequence ACAGCTGCAA on the vector DNA strand to ACGCTGCAA. These substitutions caused an exchange of two alanine to proline residues. The upper primer encoded the sequence GGA GTC ACC ATG GCT GAT CAA GGT CAC ATG (modified by three operators; Ref. 33), by excising them from the pSK vectors as KpnI/XbaI fragments and inserting them in pBIBInHcyTX (32), cleaved with KpnI and XbaI. The constructs were transformed into Agrobacterium tumefaciens GV3101 competent cells containing the pMP90 vector (34). Transformation of tobacco plants was performed using the Agrobacterium leaf disc technique as described previously (35).

Northern Blot Analysis—Total RNA was purified from tobacco leaves using the RNeasy plant kit (Qiagen). RNA concentrations were determined spectrophotometrically, and 15 μg of RNA for each sample were electrophoresed on denaturing agarose gels and transferred to nylon membranes (Schleicher & Schuell; Ref. 36). RNA blots were prehybridized in 50% formamide, 50 mM sodium phosphate, pH 6.8, 1% SDS, 10% dextran sulfate sodium salt, and 1 mM NaCl at 1 h at 42 °C. Hybridization was performed in the same solution with 100 μg/mL salmon sperm carrier DNA and 32P-labeled probe for 14–16 h at 42 °C. The probe was made by the random-priming method (37) with the Megaprime DNA labeling system (Amersham Pharmacia Biotech). The cDNA probes used for Northern blot hybridizations were: tobacco Nt103, a 1-kilobase pair full-length cDNA encoding the auxin-inducible gene Nt103 (38); tobacco IEGT, a 701-bp cDNA fragment of the immediate-early SA-induced glucosyltransferase (39); a 715-bp fragment of the tobacco PArA gene (40); tobacco PR-1a, a 531-bp fragment of pathogenesis-related protein cDNA isolated from tobacco bath infection (41) or by Dr. K. Pawlowski, University of Goettingen, Goettingen, Germany); and a 1-kilobase pair pea 18 S rRNA probe (Ref. 42; kindly provided by Dr. S. Koehne, University of Bielefeld, Bielefeld, Germany). Washing was performed with 2× SSC, 0.1% SDS at 65 °C for two 30-min periods and with 0.5× SSC, 0.1% SDS at 65 °C for 20 min as required. Quantification of RNA levels was accomplished by Bioimager analysis (Fujix BAS 1000; Fujif). Hybridization signals of Nt103, IEGT, ParA, and PR-1a were normalized using either the 18 S rRNA or the actin mRNA obtained by subsequent hybridization of the same filter.

RESULTS

The biotinylated DNA fragment was immobilized on streptavidin-coated, paramagnetic beads (Dynabeads™ M-280 streptavidin; Dynal) as follows. After prewashing the Dynabeads in 1× B&W buffer (2 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) according to the manufacturer’s specifications, 1 mg of beads were resuspended in 0.5× B&W buffer containing 200 ng of the purified 5× as-1 PCR fragment. Coupling occurred during incubation for 30 min at room temperature with gentle rotation of the tubes. Afterward, the beads were washed in 1× B&W buffer and equilibrated in binding buffer (25 mM Hepes-KOH, pH 7.6, 10% glycerol, 10 mM MgCl2, 0.2 mM CaCl2, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonlfluoride). For purification of SARP, all incubations were done at room temperature and with gentle rotation of the tubes. Cellular extract prepared from BY-2 cells was diluted 1:4 in binding buffer and equilibrated in binding buffer. The initial cellular extract mixture was added to 1 μg of Dynabeads (coupled with 0.2 ng of 5×as-1 fragment) per μg of cellular protein, and binding reaction was extended to 20 min. Afterward, beads with the adsorbed DNA-binding proteins were separated from the supernatant and washed twice for 10 min with 1 volume of binding buffer (supplemented with 0.5 mg/mL BSA) including the same concentration of poly(diLIC) and G-box oligonucleotide as used in the binding reaction. Finally, elution of DNA binding proteins was performed by twice adding 0.125 volume of elution buffer (binding buffer supplemented with 2 mM NaCl; if the eluted fraction was used for EMSAs, 0.5 mg/mL BSA was added) to the beads (eluted fractions 1 and 2; elution time, 15 and 10 min, respectively). The initial cellular extract and the eluted fraction 1 were analyzed for specific as-1 and G-box binding activities in EMSAs. Because the eluted fraction 1 is twice as concentrated compared to the initial extract, we added 1 volume of cellular extract corresponding to 5 μg of protein and 0.5 volume of eluted fraction 1 to the binding reaction. Because of the very low protein content, 10 μg of BSA was included in binding reactions with the eluted fraction. Eluted fractions 1 and 2 from initially 3.4 mg of cellular proteins were collected, dialyzed against water, freeze-dried, and resuspended in 25 μl of denaturation buffer (8 M urea, 0.1 M NaH2PO4, 10 mM Tris-HCl, pH 8). 2.5 μl of the sample were separated on a 10% SDS-PAGE and subjected to Western blot analysis.

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to that of SARP from leaf extracts (Fig. 1c). The extract was loaded onto magnetic beads coated with a multimer of the as-1 element. To deplete the extract from GBFs, loading of the beads was done in the presence of an oligonucleotide encoding the G-box, followed by washing steps with the same oligonucleotide. After elution of the complex with 2 M NaCl, −65% of SARP were regained, whereas the amount of GBFs had decreased considerably (Fig. 2, a and b). In order to determine whether the enrichment procedure had changed the composition of SARP, supershift analysis of the eluted fraction was performed with the antibodies against TGA1a, TGA2.1, and TGA2.2. Again, low amounts of the complex were supershifted with the anti-TGA2.1 serum, whereas the majority of the complex was supershifted with anti-TGA2.2 serum, indicating that none of the two factors was preferentially lost (Fig. 2c). Next, enriched SARP was loaded on a Western blot along with recombinant TGA1a, TGA2.1, and TGA2.2 (Fig. 3). Using the antibody directed against the basic DNA-binding domain of TGA factors, one prominent and four weaker signals were detected. The prominent band was slightly smaller than recombinant TGA2.2, which is due to a three-amino acid N-terminal extension of the recombinant protein. Thus, the dominant component of SARP is TGA2.2. As the prominent band is stronger than the total of the other bands, we conclude that a large portion of SARP is a TGA2.2 homodimer. Probing the blot with the TGA2.2 antibody confirmed that this band is indeed TGA2.2. The ratio of the intensity of the signal of SARP-derived TGA2.2 and recombinant TGA2.2 was very similar to the ratio detected with the antibody against the basic domain, indicating that no additional protein of the same size co-migrated with SARP derived TGA2.2. A faint band migrating above TGA2.2 cross-reacted with the TGA2.1 antibody. As this protein runs considerably faster than recombinant TGA2.1, we call it TGA2.1-related activity. The low abundant proteins run-

FIG. 1. Supershift analysis of ASF-1 and SARP. a, Nuclear extracts were prepared from tobacco leaves. For each lane, 10 μg of nuclear extracts were added to the binding reaction with a radiolabeled 77-bp-long as-1 encoding fragment. The ASF-1 complex is characterized by two protein-DNA complexes representing single and double occupancy of the two TGACG motifs. At the top of each lane is indicated whether the preimmune serum (pTGA2.1, pTGA2.2, pTGA1a) or antisera (αTGA2.1, αTGA2.2, αTGA1a) were added (1 μl). The mobility of the TGA2.2 as-1 complex is shown using in vitro translated TGA2.2 protein. b, Cellular extracts were prepared from tobacco leaves (14). For each lane, 20 μg of cellular extracts were added to a 77-bp-long radiolabeled as-1 encoding DNA fragment. The SARP complex is characterized by two protein-DNA complexes representing single and double occupancy of the two TGACG motifs. At the top of each lane is indicated whether the preimmune serum (pTGA1a) or antisera (αTGA1a, αTGA2.2, αTGA1a) were added (1 μl). c, Cellular extracts were prepared from tobacco cell line BY-2 4 days after subculturing. For each lane, 5 μg of cellular extracts were added to a 77-bp-long radiolabeled as-1 encoding DNA fragment. At the top of each lane is indicated, whether the preimmune serum (pTGA1a) or antisera (αTGA2.1, αTGA2.2, αTGA1a) were added (1 μl). Relative amounts of ASF-1 or SARP and supershifted complexes are indicated below the lanes. Numbers were average values from three independent extracts (a and b) and from one extract (c).

FIG. 2. EMSAs and supershift analysis of affinity-purified SARP. a, EMSAs using the 250-bp radiolabeled 5×as-1 fragment as a probe. Loading was done after addition of 5 μg (1.06 μl) of cellular extract, prepared from BY-2 cells, and 0.53 μl of SARP, purified using magnetic beads coated with a multimerized as-1 element (fraction eluted with high salt). b, EMSAs using the 55-bp radiolabeled G-box as a probe. Loading of lanes was done as in a. The ratio of TGA factors to GBFs increased considerably due to depletion of GBFs. c, supershift analysis of affinity-purified SARP to demonstrate that the composition of SARP did not change during the affinity purification. 0.53 μl of the eluted proteins were added to the binding reaction containing a 77-bp radiolabeled as-1 encoding fragment. At the top of each lane is indicated which of the three antisera (αTGA1a, αTGA2.2, αTGA1a) was added (1 μl).
ning below TGA2.2 interacted with none of the specific antibodies. We do not know whether these proteins constitute further unidentified TGA factors or degradation products missing the N termini of TGA2.2 and/or TGA2.1.

Functional Analysis of TGA2.2 in Planta—Having defined TGA2.2 as a major component of ASF-1 or SARP, we analyzed its impact on as-1-encoding potential target genes. Two types of transgenic plants expressing different TGA2.2 alleles were generated (Fig. 4a). The first one encodes the wild-type allele under the control of the cauliflower mosaic virus 35 S promoter. The second type of transgenic plants contains the same chimeric gene with only two point mutations in the conserved DNA-binding domain. These mutations change two alanine residues into proline residues. As the DNA-binding domain assumes a helical structure upon binding, we expected that the introduction of the helix-breaking amino acid proline should abolish DNA binding while maintaining heterodimerization properties. TGA mutants with defective DNA-binding domains have been shown previously to specifically suppress the activity of endogenous TGA factors by forming inactive heterodimers (32, 45). This was confirmed by co-translation of mutated TGA2.2 (TGA2.2trd) with either TGA2.2 or TGA1a and subsequent gel shift analysis (Fig. 4, b and c). As expected, TGA2.2trd is deficient in DNA binding and reduces binding of TGA2.2 and TGA1a.

Transformants were phenotypically normal, independent of the level of transgene expression. Fig. 5a depicts the mRNA levels of the two highest expressing plants of each construct, which were chosen for further analysis. Plants transformed with the chimeric TGA2.2 gene expressed 2-fold less transgenic mRNA levels than the two highest expressing TGA2.2trd plants, indicating counterselection against high levels of TGA2.2. This notion is supported by the finding that only two strong TGA2.2 overexpressors were found in 28 primary transformants analyzed, whereas five strong TGA2.2trd overexpressors were found in 7 primary transformants. Western blot analysis with the TGA2.2 antibody confirmed the synthesis of transgenic TGA2.2 proteins (Fig. 5b). Western blot analysis using an antibody directed against glutamine synthetase (kindly provided by Dr. Tischner) indicated that the quality of the different extracts was comparable (Fig. 5c). Subsequently, these extracts were subjected to supershift analysis. Transgenic plants overexpressing TGA2.2 revealed a ~2.5-fold in-

![FIG. 3. Western blot analysis of affinity-purified SARP. Middle panel, purified SARP was dialyzed against water, freeze-dried, and resuspended in denaturation buffer. The sample (2.5 μl, eluted fraction) was electrophoretically separated on a 10% SDS gel along with crude extracts of E. coli (diluted 1:20) expressing TGA2.2 (0.36 μl), TGA1a (2 μl), and TGA2.1 (1.25 μl), respectively. Detection of bZIP proteins was done by using an antibody against a synthetic peptide encoding the conserved DNA-binding domain. Lane 6 is a longer exposure of lane 7. Right panel, the filter was stripped and probed with an affinity-purified TGA2.2 antibody to prove that the major band of the eluted fraction of lane 6 is TGA2.2. Left panel, the filter was stripped again and probed with an affinity-purified TGA1.1 antibody to identify the size of the TGA1.1-related activity. Lane 5 is a longer exposure of lane 1. Arrows point to the five bands detected by the general antibody anti-TGA; the double arrow points to the TGA2.1-related activity. Numbers between the middle and the right panel indicate the number of amino acids of the recombinant proteins.

![FIG. 4. Construction of a dominant negative TGA2.2 allele. a, schematic presentation of the dominant negative TGA2.2 allele (TGA2.2trd). The sequence of the basic DNA-binding domain is shown, as well as point mutations introduced by PCR. b, SDS-PAGE of in vitro 35S-labeled TGA factors. The cDNA sequences of tobacco TGA1a, TGA2.2, and TGA2.2trd were transcribed under the control of the T7 promoter. Mixtures of equal amounts of RNAs were translated using the TNT reticulocyte lysate (Promega), containing [35S]methionine. 5 μl of the in vitro translation reaction were loaded on each lane to estimate the efficiency of protein synthesis. c, EMSA of in vitro translated TGA factors. A 77-bp-long 32P-labeled DNA fragment encoding the as-1 element was used as a probe.}
crease in SARP activity, whereas transgenic plants encoding TGA2.2trd showed a ~2.3-fold decrease in SARP activity (Fig. 5d). Supershift analysis did not indicate a dramatic change in the composition of SARP. The anti-TGA2.1 serum recognized 20% of SARP in wild-type and TGA2.2trd plants. In plants overexpressing TGA2.2, only 10% of SARP was recognized by the anti-TGA2.1 serum, indicating that the relative levels of SARP correlated with altered expression of potential target genes. SA-responsive genes are grouped into “immediate early” and “late” genes according to the kinetics of the induction (8), Nt103 (a glutathione S-transferase; Ref. 46), IEGT (a gene with homology to a glucosyltransferase; Ref. 39) and ParA (a gene coding for a nuclear localized gene product with remote similarity to glutathione S-transferases; Ref. 47) belong to the immediate early genes, which are transiently induced after 2–3 h of SA treatment. The respective promoters encode as-1-like elements, which are recognized by ASF-1 (48, 49).

Next we examined whether changing SARP levels also correlated with altered expression of potential target genes. SA responsiveness of the immediate early genes Nt103, IEGT, and ParA (Fig. 8). 2,4D induction of the IEGT promoter was not as strongly affected by the transgene. The most striking effects were observed with Nt103. Elevated levels of SARP correlated with a 2.9- and 4.7-fold increase in SA responsiveness, whereas reduction of SARP correlated with a 5.5- and 5.6-fold decrease of the response (Fig. 7). The IEGT promoter was not as strongly affected by the transgenes as the Nt103 promoter. In contrast, overexpression of TGA2.2 had no effect on the expression of ParA or PR-1a, although expression of TGA2.2trd decreased SA inducibility of these promoters ~5-fold.

Next we examined whether changing SARP levels also correlated with effects on 2,4D inducibility of the immediate early genes Nt103, IEGT, and ParA (Fig. 8). 2,4D induction of Nt103 was 1.7- and 2.0-fold higher in TGA2.2-overexpressing plants and 3.5- and 4.0-fold lower in TGA2.2trd-expressing plants. The response was specific for 2,4D and did not occur with 2,6D. 2,4D inducibility of IEGT was only slightly affected (2-fold) in TGA2.2trd plants, but not in TGA2.2-overexpressing plants.

Fig. 5. Expression of TGA2.2 alleles in transgenic plants. a, Northern blot analysis of transgenic plants expressing either TGA2.2 or TGA2.2trd. 15 µg of total RNA were loaded in each line. Two independent transformants with highest expression levels are shown. Hybridization was done with a TGA2.2 probe. Arrows point to the endogenous (upper arrow) and the transgenic (lower arrow) transcript. b, Western blot analysis of transgenic plants expressing either TGA2.2 or TGA2.2trd, using an antibody directed against the N terminus of TGA2.2. 100 µg of cellular extracts (14) were loaded. The arrow marks the specific band. c, Western blot analysis of transgenic plants expressing either TGA2.2 or TGA2.2trd, using an antibody directed against glutamine synthetase to control the quality of the extract. 40 µg of cellular extracts (14) were loaded. d, EMSA and supershift analysis of transgenic plants expressing either TGA2.2 or TGA2.2trd. Cellular extracts were prepared from tobacco leaves of either untransformed plants or plants overexpressing TGA2.2 or TGA2.2trd. For each lane, 20 µg of cellular extracts were added. Addition of 1 µl of the different antisera is indicated at the top (αTGA2.1, αTGA2.2, αTGA1a). Radiolabeled probe was the 77-bp as-1 encoding DNA fragment. The panel below the lanes indicates the percentage of supershifted SARP (see also Fig. 1).
were floated on 1 mM SA or 1 mM HBA for 2 and 3 h before extraction of RNA. 15 m g of PR-1a having a value of 1.0.

DISCUSSION

To further elucidate the regulation of the as-1 element, we analyzed the composition of a nuclear (ASF-1) and a cellular (SARP) as-1 binding activity previously identified in leaf extracts (13, 14). The major component of both complexes is a TGA2.2 homodimer. A TGA2.1-related gene product was also detected, albeit to significantly lower amounts. Overexpression of TGA2.2 or a dominant negative TGA2.2 mutant led to increased and decreased SARP levels, respectively. The activities of four different SA-inducible specificity. The composition of these complexes is particularly interesting with regard to the elucidation of the regulatory mechanism leading to SA-inducible gene expression. According to a model suggested by Jupin and Chua (14), SARP associates with a hypothetical protein (SAI) in the absence of SA. This SAI protein is believed to interfere with binding of SARP to as-1.

**Fig. 7.** Expression levels of SA-inducible target genes in transgenic plants overexpressing either TGA2.2 or TGA2.2trd. Leaves were floated on 1 mM SA or 1 mM HBA for 2 and 3 h before extraction of RNA. 15 μg of total RNA were loaded. Blots were hybridized with probes for Nt103, IEGT, ParA, and 18 S rRNA as indicated. For analysis of PR-1a, leaves were floated on 1 mM SA for 12 and 24 h. Northern blots are only shown for one of the two independent transformants. Transcript levels were quantified by Bioimager, and values were corrected for small variations in loading by standardization to the 18 S rRNA values. Numbers indicate the amount of increase or decrease of expression levels of two independent transgenic plants, with the control plants having a value of 1.0.

Expression Pattern and Binding Activity of TGA2.2—Expression levels of pre-existing regulatory proteins often increase under inductive conditions. Therefore, we analyzed whether the expression level of TGA2.2 changes upon treatment of plants with either SA or 2,4D. SA treatment for up to 36 h under inductive conditions. Therefore, we analyzed whether the expression level of TGA2.2 changes upon treatment of plants with either SA or 2,4D. SA treatment for up to 36 h.

**TGA2.2 Is the Main Component of ASF-1 and SARP—**Two different protocols for the enrichment of as-1-binding proteins from leaf extracts have been reported. Originally, ASF-1 was communicated as a nuclear DNA binding activity (13). Later, Jupin and Chua (14) described a cellular factor binding to as-1, which they called SARP. SARP and ASF-1 were not directly compared with respect to electrophoretic mobility or binding with the anti-TGA2.2 serum. The composition of ASF-1 did not change upon SA or 2,4D treatment of leaf disks (data not shown).

Dissociation of SARP from SAI, which depends on phosphorylation of either of the two partners, leads to a 3-fold increase of SARP activity in leaves. Thus, the knowledge of the composition of SARP identifies one of the two interacting partners, yielding a promising strategy for a two-hybrid screen to identify SAI. Eighty percent of ASF-1 and SARP were supershifted with the anti-TGA2.2 serum. The composition of ASF-1 did not change upon SA or 2,4D treatment of leaf disks (data not shown). To decide whether TGA2.2 is represented in the complex as a homodimer or a heterodimer, SARP was analyzed by Western blot analysis after affinity purification against as-1-encoding oligonucleotides attached to a solid matrix. An antibody against the conserved basic domain of TGA-factors identified TGA2.2 as the dominant protein of SARP, indicating that a large fraction of TGA2.2 in SARP must be represented as a homodimer. This is consistent with results reported by Jupin and Chua (14); Southwestern blot analysis of SARP had indicated that a 40-kDa protein, which is smaller than TGA1a but immunologically related, is the main component of SARP.

The calculated molecular mass of TGA2.2 (36.2 kDa) is close to the size reported by Jupin and Chua (14). As TGA1a, PG13, and TGA2.1 have molecular masses of 43.3, 43.3, and 50.0 kDa respectively, it seems very likely that Jupin and Chua (14) and our group have indeed analyzed the same activity. Unfortunately, the 3-fold increase of SARP binding activity upon SA treatment was not reproducible in our hands (data not shown). As we obtain considerable amounts of SARP in extracts of untreated leaves, the most likely explanation is that SAI is so labile that its activity is readily lost during the extraction. This is consistent with the finding that we could also not reproduce the formamide induced increase in SARP activity, which was interpreted to lead to the dissociation of the SARP-SAI complex (14). TGA2.2 may still be a good bait to isolate SAI in a yeast two-hybrid system.

A TGA2.1-related Activity Is Present in Minor Amounts in ASF-1 and SARP—Only minor amounts of ASF-1 (10%) or SARP (20%) were recognized by the anti-TGA2.1 serum. As the respective protein is significantly smaller than the recombinant TGA2.1 protein, TGA2.1 may still be a good bait to isolate SAI in a yeast two-hybrid system. This is consistent with results reported by Jupin and Chua (14). As TGA1a, PG13, and TGA2.1 have molecular masses of 43.3, 43.3, and 50.0 kDa respectively, it seems very likely that Jupin and Chua (14) and our group have indeed analyzed the same activity. Unfortunately, the 3-fold increase of SARP binding activity upon SA treatment was not reproducible in our hands (data not shown). As we obtain considerable amounts of SARP in extracts of untreated leaves, the most likely explanation is that SAI is so labile that its activity is readily lost during the extraction. This is consistent with the finding that we could also not reproduce the formamide induced increase in SARP activity, which was interpreted to lead to the dissociation of the SARP-SAI complex (14). TGA2.2 may still be a good bait to isolate SAI in a yeast two-hybrid system.

**Plants with Increased or Decreased SARP Levels Were Generated by Overexpression of TGA2.2 and a Dominant Negative TGA2.2 Mutant—**Expression of TGA2.2 or a dominant negative TGA2.2 mutant led to increased and decreased SARP levels, respectively. The activities of four different SA-inducible

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promoters containing as-1-like elements were analyzed in these plants. Three of these promoters respond with identical kinetics both to SA and 2,4D as the isolated as-1 element (Fig. 6), suggesting that they are subject to the same control mechanism. The analysis of transgenic plants expressing either TGA2.2 or a dominant negative TGA2.2 mutant served to investigate the following questions. 1) Is TGA2.2 limiting for the expression of endogenous plant genes encoding the as-1 element? 2) Does overexpression of TGA2.2 lead to constitutive expression of target genes, e.g. by outcompeting the postulated inhibitory factor SAI? 3) Do SA- and 2,4D-activated signal transduction chains activate TGA2.2?

Analysis of SA-inducible Gene Expression in Transgenic Plants Expressing TGA2.2 or a Dominant Negative TGA2.2 Mutant—Out of the four potential target promoters analyzed, the Nt103 and the IEGT promoter were clearly affected by both TGA2.2 alleles. Overexpression of TGA2.2 led to a 3–4-fold higher SA inducibility. This indicates that TGA2.2 can be activated by SA and that it helps to activate the promoter either as a homodimer or a heterodimer. Thus, the answer to question 1 asked above is that TGA2.2 can be limiting for the expression of endogenous plant genes. No significant increase in uninduced or mock-induced samples was observed, answering question 2 asked above. In transgenic plants expressing the dominant negative TGA2.2 derivative, gene expression was significantly reduced (5–10-fold). This indicates that factors heterodimerizing with TGA2.2, i.e. endogenous TGA2.2 and/or other members of the TGA family, are indeed involved in gene expression of these defense genes. Alternatively, other regulatory components might bind to the nonfunctional TGA dimer becoming thus limiting for the events happening at the promoter. At this point, we cannot decide, whether the observed effects are due to direct binding of TGA2.2 to the Nt103 and the IEGT promoter, or due to binding of TGA2.2 to a promoter driving a second trans factor. We consider the latter possibility to be less likely, because Nt103 expression does not require de novo protein biosynthesis and TGA2.2 would thus have to induce this hypothetical trans factor in the absence of SA.

Increased TGA2.2 levels did not modify the expression of ParA or PR-1a in response to SA. In contrast, expression of TGA2.2trd led to decreased transcription from these promoters, supporting the importance of this family of transcription factors for SA-inducible transcription. As these promoters display stronger activities than the promoters driving Nt103 and IEGT, other factors might become limiting to gain a further increase upon overexpression of TGA2.2. Alternatively, a different TGA factor might activate these promoters. In the case of the PR-1a promoter, an indirect effect must be discussed, as activation depends on de novo protein synthesis. In this case, reduced levels of functional TGA proteins might not only affect the promoter through the PR-1a-encoded as-1 element, but might also influence the expression of a primary response component, which must be synthesized a priori to obtain maximal promoter activity.

Analysis of 2,4D-inducible Gene Expression in Transgenic Plants Expressing TGA2.2 or a Dominant Negative TGA2.2 Mutant—as-1-like elements have also been called multiple stimuli-responsive elements, as they are responsive to other chemical stimuli like 2,4D, several heavy metals, and methyljasmonate (50). Upon 2,4D treatment, Nt103 levels were induced more strongly in TGA2.2-overexpressing plants as compared with wild-type plants. This implies that TGA2.2 can also be activated by 2,4D and answers question 3 asked above. In TGA2.2trd-expressing plants, Nt103 and IEGT were to a lesser extent 2,4D-inducible than in wild-type plants. The observed effects of the transgene were less pronounced than upon SA treatment. Several explanations for the lower sensitivity of these promoters to changing TGA levels after 2,4D treatment in comparison to SA treatment can be envisioned. 1) These promoters might be occupied by a different combination of non-TGA-related trans-factors upon 2,4D treatment than upon SA treatment. This might enhance the affinity of TGA factors to these promoters, making them less responsive to altered levels of functional TGA factors. Indeed, promoter activities are higher upon 2,4D treatment than upon SA treatment, supporting the notion that both responses are modulated by different trans-acting factors interacting with TGA factors. 2) A different heterodimer between TGA2.2 and another TGA factor might be responsible for 2,4D induction as compared with SA induction. As outlined above, SARP has been characterized as an in vitro as-1 binding activity, which selects for the most stable complex. It might well be that a different combination of TGA factors assembles on different promoters upon different stimuli in vivo. In this case, we would have to postulate that TGA2.2trd heterodimerizes less efficiently with this factor as compared with the situation upon SA induction. 3) Other limiting factors...
allow only a 2-fold (N103) or no (IEGT) increase in gene expression. Any of these explanations might also be relevant for the failure of the transgenics to alter 2,4-D-induced expression from the ParA promoter. TGA factors may even be dispensable for ParA.

In conclusion, the composition of SARP as well as the analysis of transgenic plants expressing TGA2.2 or TGA2.2trd suggests that TGA2.2 is involved in activating target promoters. TGA factors may even be dispensable for ParA. However, TGA2.2 does not encode a potential activation domain, as shown by its heterologous expression in yeast (25). Thus, we favor the hypothesis that TGA2.2 acts in combination with different members of the TGA family, one candidate being TGA2.1.

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Tobacco Transcription Factor TGA2.2 Is the Main Component of as-I-binding Factor ASF-1 and Is Involved in Salicylic Acid- and Auxin-inducible Expression of as-I-containing Target Promoters

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