Regulation of DNA Binding and trans-Activation by a Xenobiotic Stress-activated Plant Transcription Factor*

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As-1-type cis-elements augment transcription of both nuclear and pathogen genes in response to stress and defense cues in plants. Basic/leucine zipper proteins termed "TGA factors" that specifically bind as-1 elements are likely candidates for mediating these transcription activities. Our earlier work has shown that 2,4-dichlorophenoxyacetic acid-induced xenobiotic stress enhances trans-activation by a chimeric fusion protein of the yeast Gal4 binding domain and TGA1a, a TGA factor of tobacco. Here we demonstrate that xenobiotic stress also enhances the ability of native TGA1a to bind as-1 and activate transcription from a known target gene. In addition, the previously identified xenobiotic stress-responsive domain of TGA1a was found to inhibit this factor's trans-activation potential by a mechanism that appears to involve stimulus-reversible interactions with a nuclear corepressor protein. Results from these and other studies can now be placed in the context of a working model to explain basal and xenobiotic stress-induced activities of TGA1a through its cognate cis-acting element.

As-1-type elements contribute to the expression of both pathogen and nuclear genes in plants (1–9). When inserted upstream of a minimal promoter and β-glucuronidase reporter gene, these elements drive β-glucuronidase expression predominantly in primary and lateral root tips of tobacco and Arabidopsis seedlings, and in the root and shoot vascular system of older plants (2, 10, 11). This transcription activity is further augmented in response to plant defense hormones (e.g. salicylic and jasmonic acids), wounding, and xenobiotic stress, thus indicating that diverse stimuli affect the activity of one or more cognate transcription factors through these elements.

A number of genes have been cloned that encode for as-1-binding proteins, both in the same and different plant species. These "TGA factors" share considerable homology and belong to the basic/leucine zipper (bZIP) family of transcription factors. Efforts to understand the contributions of TGA factors to as-1-dependent transcription will require knowledge of their cellular distribution and molecular properties. To this end, we initially chose to study TGA1a because close homologues of this tobacco TGA factor exist in other plants, suggesting a conserved and perhaps important biological role. In prior studies, we found that a chimeric protein comprised of the yeast Gal4 binding domain and TGA1a could potentiate transcription through the GAL4 cis-element in response to xenobiotic stress. These data implicate TGA1a in the expression of plant genes involved in chemical defense (12). Consistent with this notion, TGA1a transcripts and protein are preferentially coexpressed in root meristem cells with transcripts of as-1-regulated genes (e.g. GNT35) (11) that encode for type III glutathione S-transferase (GST) isoenzymes. Although the biological function of these as-1-regulated GSTs is unknown, this class of enzymes has been specifically implicated in xenobiotic detoxification and resistance in plants (13–15).

Gain of function assays with the yeast Gal4 binding domain led to the identification of specific TGA1a domains involved in basal and xenobiotic stress-activated transcription (12). By this assay, it was shown that the amino-terminal (NT) domain (residues 1–142) of TGA1a confers constitutive trans-activation, whereas the carboxyl-terminal (CT) domain (residues 142–373) of this factor largely enhances transcription in response to xenobiotic stress. Here we show that xenobiotic stress rapidly and transiently affects the ability of native TGA1a to bind its cognate as-1 element and to activate transcription through a mechanism involving stimulus-reversible repression and this factor’s regulatory CT domain. Additional evidence suggests that this regulatory mechanism is likely to occur through stimulus-reversible interactions of the CT domain with a putative corepressor protein.

EXPERIMENTAL PROCEDURES

Tobacco Suspension Cell Cultures

Cultures (100 ml) of tobacco BY-2 suspension cells were grown and maintained in flasks at 28 °C in the dark as described previously (12). For the preparation of labeled nuclear proteins, cells were cultured for 4 days and adjusted to a packed volume of 50%, and 4 ml of the cell culture were transferred to each of 5 wells in an 8-well culture plate (Costar). After 24 h, 200 μCi of [35S]methionine (6000 Ci/mmol; PerkinElmer Life Sciences) were added to each sample, which was then incubated for an additional 20 h. Where indicated, cells were exposed to xenobiotic stress induced by treatment for 0–8 h with 100 μM 2,4-dichlorophenoxyacetic acid (2, 4-D) in 0.1% ethanol carrier, collected by vacuum filtration, frozen in liquid nitrogen, and stored at –80 °C.

Effector and Reporter Gene Constructs

Reporter gene constructs and the effector construct of FLAG epitope-tagged TGA1a were prepared as described previously (12). TGA1aACT, which lacks CT residues 142–373 of TGA1a, was synthesized through PCR using TGA1a as template and the following set of primers: 5′-ACTTGAGAATTTCTCTCCAACTACGACCCATTT-3′ and 5′-AAAACTGATTCGCTTCTAGTTGTGT-3′, corresponding to residues 1–142. TGA1a-CREBACT was made by separately PCR-amplifying residues 1–142 of TGA1a, as described above, and two leucine zipper repeats

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§ The abbreviations used are: bZIP, basic/leucine zipper; 2,4-D, 2,4-dichlorophenoxyacetic acid; GST, glutathione S-transferase; NT, amino-terminal; CT, carboxyl-terminal; PCR, polymerase chain reaction; CREB, cAMP-response element-binding protein; CAT, chloramphenicol acetyl transferase; PAGE, polyacrylamide gel electrophoresis.
(residues 311–315) of CREEB using the primers 5′-AAAGGCTATG-TCAAGAGTGAACAGACTG-3′ and 5′-AAATCCGATCTGCTC-TAACTGCTT-3′ with Rous sarcoma virus CREEB as template. Full-length PCR products were gel-purified, combined, and amplified with primers corresponding to the 5′ and 3′ termini of TGA1a and CREEB leucine zipper sequences, respectively, to yield a single intact translational fusion product. TGA1a-CREEB also required a more complex subcloning strategy involving separate and sequential PCR amplifications. TGA1a-CREEBΔCT was used as template with the following primers to generate fragment 1: 5′-ACITGGGAATTCTCTCACGACT-CAATT-3′ and 5′-GTCTTTAACGTTTTTGAATCAATGTGATG-3′. TGA1a was used as template with the following primers to generate fragment 2: 5′-ACATTGGTAGACCAGGTAAACATTGAGCTGATGATGCAGATGCT-3′ and 5′-TTATTCATGCTACGAGGCTCAGCTACATGAGG-3′. Both DNA fragments were agarose gel-purified and combined in equal amounts to serve as template for PCR reactions with the following primers: 5′-ACITGGGAATTCTCTAACGACTCAATTTTCAATTTT-3′ and 5′-TTATTCATGCTACGAGGCTCAGCTACATGAGG-3′. For each of the TGA1a mutants, the subterminal primer used to make the final construct contained a TAA stop codon in-frame after the last TGA1a residue. All PCR reactions were run as described, and the resultant products were treated with proteinase K, digested with EcoRI and ClaI, agarose gel-purified, and ligated into cognate restriction sites in KS-FLAG vector (12). The TGA1a constructs were sequenced and tested for their ability to express the expected FLAG epitope-tagged protein using a coupled in vitro transcription/translation system (Promega). The FLAG-tagged cDNA was excised with BsmHI and ClaI, blunt-ended with Klenow enzyme and deoxynucleotide triphosphates, and subcloned into the plant expression vector pMON9999 as described previously (12).

Protoplast Transient Transfection Assay

Protoplasts were prepared from BY-2 suspension culture cells and transfected with the indicated plasmid reporter and effector DNAs as described previously (12). Transfected protoplasts were collected by brief centrifugation and then treated with lysis buffer according to the manufacturer’s instructions (Promega). Lysates were assayed for reporter gene activity by luciferase or chloramphenicol acetyl transferase (CAT) enzyme assays (12, 16). To correct for differences in transfection efficiency, reporter gene activity of -90-CAT was normalized to that of CHS-LUC, a luciferase reporter gene that is transcribed from a bean chalcone synthase promoter (17). Data shown are the mean and standard error from three or more independent experiments.

Nuclear Run-on Assay

Six days after transfer into fresh medium, BY-2 cells were incubated with 100 μM 2,4-D in 0.1% ethanol carrier for 0–8 h, collected by vacuum filtration, frozen in liquid nitrogen, and stored at −80 °C. Nuclei were isolated essentially as described by Droge-Laser et al. (18). nascent transcripts from these nuclei were radiolabeled as described by Lawton and Lamb (19) and isolated according to the work of McKnight and Palmiter (20). Labeled transcripts (~4 × 10^6 cpm) were hybridized as described by Lawton and Lamb (19) against nitro-immunized full-length cDNAs (200 ng) of the tobacco GNT35 and TGA1a genes. Radiolabeled transcripts with hybridization signals were quantified with a PhosphorImager (Molecular Dynamics).

Preparation of Nuclear Extracts

BY-2 cells were ground with a mortar and pestle to a fine powder under liquid nitrogen. Nuclear proteins were extracted from this material as described previously (7), followed by dialysis against two changes of 500 ml of dialysis buffer (20 mM HEPES, pH 7.9, 25 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, and 1 mM dithiothreitol). Dialyzed extracts were clarified at 5,000 × g for 5 min, divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C.

Preparation of Recombinant TGA1a

Recombinant TGA1a was synthesized using a coupled Trx/T7 transcripition/translation system (Promega) with pBluescript KS vector containing full-length cDNA of TGA1a as template (12).

DNA-affinity Chromatography

Preparation of Labeled Nuclear Extracts—BY-2 cells cultured for 6 days were incubated with 200 μCi/ml [35S]methionine (6000 Ci/mmol; PerkinElmer Life Sciences) for 24 h and then treated for 1 h with either 100 μM 2,4-D in 0.1% ethanol carrier (xenobiotic stress) or 0.1% ethanol carrier alone (mock). Labeled nuclear protein extracts were prepared as described above. Preparation of DNA-affinity Resins—Oligonucleotide concatamers of wild-type or mutant as-1 sequences were coupled to cyanogen bromide-activated Sepharose according to the manufacturer’s instructions (Amersharm Pharmacia Biotech). The coupling efficiency of these oligonucleotide-DNA complexes was determined by DCTA agarose gel electrophoresis. For each coupling reaction, 125 ng of immobilized DNA were used. Immobilized DNA was equilibrated in binding buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 0.2 mM EDTA, 25 μg/ml poly(dIdC), 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiotreitol, and 20% (v/v) glycerol) for 15 min on ice.

Assay Conditions—Wild-type or mutant immobilized DNAs were then incubated with 10^5 cpm [35S]methionine (Promega) and rabbit reticulocyte lysate for 30 min on ice. Translation was initiated using rabbit reticulocyte lysate mixture containing 20 μl of Laemmli loading buffer and denatured by boiling, fractionated by SDS-PAGE on a 10% running gel, and detected by fluorography with En3hance according to the manufacturer’s instructions (PerkinElmer Life Sciences).

Immunoprecipitation

Protein extracts were clarified by brief centrifugation at 14,000 × g and incubated on ice for 1 h with 5 μl of either preimmune sera or immune sera prepared against NT residues 57–72 of TGA1a (11). Immune complexes were recovered with 10 μl of Gammabind Plus Sepharose (Amersharm Pharmacia Biotech) in RIPA buffer solution and 2% bovine serum albumin (Sigma) during gentle mixing for 30 min on ice. Labeled protein was resuspended in 50 μl of Laemmli loading buffer denatured by boiling, fractionated by SDS-PAGE on a 10% running gel, and detected by fluorography with En3hance according to the manufacturer’s instructions (11).

GST Binding Assay

GST Fusion Proteins—PCR amplification of TGA1a and its derivatives was done using the following sets of primers and KS-FLAG as template (12): (a) for TGA1a-CT, 5′-ACITGGGAATTCTCTCACGACT-CAACCCCATT-3′ and 5′-TTATTCATGCTACGAGGCTCAGCTACATGAGG-3′; and (b) for TGA1aNT, 5′-TTATTCATGCTACGAGGCTCAGCTACATGAGG-3′. GST proteins were then cloned into pGEX-4T1 (Amersharm Pharmacia Biotech). GST fusion proteins were expressed and isolated from an E. coli BL-21 strain according to the manufacturer’s instructions (Amersharm Pharmacia Biotech). GST binding assays were done using the following sets of primers and KS-FLAG as template (12): (a) for TGA1a-CT, 5′-ACITGGGAATTCTCTCACGACT-CAACCCCATT-3′ and 5′-TTATTCATGCTACGAGGCTCAGCTACATGAGG-3′; and (b) for TGA1aNT, 5′-TTATTCATGCTACGAGGCTCAGCTACATGAGG-3′. GST protein was then purified, digested with EcoRI and Clal, and subcloned into pBluescript KS-. After amplification in the XL-1 blue strain of Escherichia coli, plasmid DNA was isolated and digested with EcoRI and XhoI. Inserts were sequenced to confirm their identity and subcloned into pGEX-4T1 (Amersharm Pharmacia Biotech). GST fusion proteins were expressed and isolated from an E. coli BL-21 strain according to the manufacturer’s instructions (Amersharm Pharmacia Biotech).

Gel-shift Binding Assay

To compare as-1 binding activities of ASF-1 from mock- and 2,4-D-treated cells, a double-stranded as-1 oligonucleotide (5′-ATCTCCCACT-GCACAGGGATTAGGCAGGCTGAC-3′) was annealed with its unlabeled complement (5′-ATCTCCCACT-GCACAGGGATTAGGCAGGCTGAC-3′). Binding reactions (25 μl) containing 20 μl HEPES, pH 7.9, 25 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 1 mM dithiothreitol, and 500 ng of nuclear protein were incubated on ice for 15 min, and then 1 μg of the radiolabeled double-stranded probe was added, and incubation was continued for 15 min. To identify as-1-bound complexes, samples were fractionated on 4% native polyacrylamide gels in 0.5× Tris-borate EDTA and analyzed by autoradiography as described previously (22). DNA binding competition assays were done with 200 ng of unla-
RESULTS

As-1-dependent Activation by TGA1a Is Repressed through Its CT Domain—Our previous work using chimeric Gal4-binding proteins led to the suggestion that the CT domain of TGA1a might function as a stimulus-reversible repressor of transcription. To further test this notion, we examined here whether removal of the regulatory CT domain from TGA1a enhanced its as-1-dependent transcriptional activity. Effector constructs of wild-type and mutant forms of TGA1a were “epitope-tagged” with the octapeptide FLAG epitope at their amino termini (Fig. 1) to facilitate immunological monitoring of steady-state amounts of these proteins. Gel-shift binding assays revealed that a mutant TGA1a factor, termed TGA1aΔCT, which lacks the entire CT domain (residues 142–373), was unable to bind as-1 (Fig. 2). This loss of function was due to the fact that CT residues appear to be essential for promoting formation of the DNA-binding dimer of TGA1a, as suggested by Katagiri et al. (22). Because the leucine zipper of TGA1a alone was unable to efficiently confer dimer formation, we pursued an alternative strategy to distinguish between dimer stabilization and repressor functions of the CT domain. This strategy involved exchanging the three heptad repeats that comprise the leucine zipper of the mammalian bZIP factor CREB. The resultant chimeric protein, TGA1a-CREBΔCT, bound as-1 as well as wild-type TGA1a (Fig. 2, lane 3). As a control, we also tested whether the same leucine zipper exchange made in full-length TGA1a affected as-1-dependent binding by this factor. Results of gel-shift binding assays indicate that this protein, TGA1a-CREB, bound as-1 to a degree that was equal to or greater than that of TGA1a (Fig. 2, lane 4). These data imply that the inability of the leucine zipper of TGA1a to efficiently promote as-1 binding is due to its weaker dimerization potential compared with that of a similar length region of the CREB zipper.

Having ascertained that the modified TGA1a factors were capable of binding as-1, we next examined their effect on as-1-dependent transcription. We found that TGA1a-CREBΔCT, which lacks the regulatory CT domain, significantly enhanced as-1-dependent transcription in the absence of xenobiotic stress, i.e. under basal conditions (Fig. 3). In contrast, neither TGA1a nor TGA1a-CREB had significant effects on basal transcription, even when increasing amounts of their respective effector genes were tested (data not shown). TGA1aΔCT, which lacks the ability to bind as-1, as expected, had little or no effect on transcription through this element.

The results above, however, might have arisen from differences in the steady-state amounts of these TGA1a proteins in transfected protoplasts. To test this possibility, we incubated transfected protoplasts with [35S]methionine and prepared nu-
clear extracts for immunoprecipitation analysis. Using this approach, we found that steady-state amounts of these TGA1a factors were generally similar among transfected protoplasts (Fig. 4). Thus, differences in the activities of these factors were not likely due to their relative abundance.

We were also interested in determining whether accessory proteins with potential regulatory activity interact with TGA1a because we previously observed that a 120-kDa protein was bound to transiently expressed TGA1a.\(^2\) Immunoprecipitation assays here confirmed this observation (Fig. 4, lane 2). We also noted that TGA1a-CREB\(\Delta\)CT, which lacks the regulatory CT domain of TGA1a, was not associated with the 120-kDa protein. This loss of activity was not due to the presence of the CREB leucine zipper in TGA1a-CREB\(\Delta\)CT because the TGA1a-CREB control factor, which contains the same zipper substitution, bound the 120-kDa protein as well as TGA1a. These observations prompted us to test whether the CT domain alone can recruit this protein.

The CT Domain of TGA1a Is Necessary and Sufficient for Stimulus-responsive Recruitment of a 120-kDa Nuclear Protein—Results here indicate that the CT domain of TGA1a inhibits trans-activation under basal conditions. This is consistent with our earlier work showing that this domain confers transcription in response to xenobiotic stress by a mechanism involving stimulus-induced de-repression (12). We thus examined here whether this regulatory domain is necessary and sufficient for recruiting the TGA1a-binding protein. In this experiment, radiolabeled nuclear proteins were incubated with GST fusion proteins of NT or CT domain polypeptides of TGA1a, washed to remove unbound material, fractionated by SDS-PAGE, and detected by fluorography (Fig. 5a). Due to

\(^2\) J. Arias, unpublished observations.

**FIG. 2.** As-1-binding by wild-type and modified TGA1a factors. a, fluorographic image of a SDS-PAGE gel showing in vitro synthesized, \(^{35}\)S-methionine-labeled TGA1a factors. Arrows indicate the expected position of the full-length proteins. b, gel-shift mobility assays were performed with \(^{32}\)P-labeled as-1 oligonucleotide as probe and 2 \(\mu\)l each of unlabeled reactions corresponding to those indicated immediately above each lane in a. P, probe only. DNA-protein complexes were detected by autoradiography.

**FIG. 3.** Effect of wild-type and modified TGA1a factors on as-1-dependent transcription. Tobacco protoplasts were transfected as described with pMON999 vector alone (mock) or with pMON999 effector DNAs encoding the indicated TGA1a factors. In addition to the as-1-regulated −90-CAT reporter gene, the CHS-LUC reporter gene was included in all transfection experiments as an internal control. After 24 h of incubation, homogenates of the protoplasts were assayed for CAT and luciferase activities. CAT activity was expressed as the percentage of conversion of nonacetylated to acetylated forms of \(^{14}\)C-chloramphenicol and then normalized to luciferase values. Values shown are the mean and S.E., expressed as the normalized percentage of conversion of chloramphenicol substrate.

**FIG. 4.** Association of wild-type and modified TGA1a factors with a 120-kDa protein. Tobacco protoplasts were transfected with pMON999 vector alone (mock) or with pMON999 effector DNAs encoding the indicated TGA1a factors and incubated overnight with \(^{35}\)S-methionine to label de novo proteins. Nuclei were isolated, and protein was extracted with RIPA buffer. The FLAG-tagged TGA1a factors were recovered from these extracts by immunoprecipitation with anti-FLAG monoclonal antibody, fractionated by SDS-PAGE, and detected by fluorography. The arrowhead indicates the position of the 120-kDa protein. Apparent molecular masses of prestained protein markers are shown in kDa.
GST proteins.

120-kDa protein.

Question. Nuclear extracts were prepared from [35S]methionine-labeled tobacco suspension cells that had been treated for 0–8 h, with either 0.1% ethanol carrier solvent (−) or 100 μM 2,4-D (+). The arrowhead indicates the expected position of the 120-kDa protein. b, the same gel stained with Coomassie Blue to detect GST proteins.

its high degree of insolubility, recombinant GST-TGA1a was not tested with this assay. Comparable amounts of each GST fusion protein were programmed in the binding reactions, as evidenced by staining of the SDS-PAGE gel with Coomassie Blue (Fig. 5b). Results show that GST-CT, but not GST or GST-NT, bound a 120-kDa protein from nuclear extracts of mock-treated cells. This interaction was not detected with extracts from cells that had been treated for 0.5 h with 2,4-D, suggesting that xenobiotic stress affects recruitment by TGA1a of a 120-kDa nuclear protein.

Binding by TGA1a to a 120-kDa Protein Is Inversely Correlated with Stimulus-induced Changes in the Rate of as-1-dependent Transcription—The results above showed that recruitment of a 120-kDa nuclear protein in vitro by recombinant CT domain protein was responsive to xenobiotic stress. Here we examined whether changes in TGA1a activity, and in its association with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are correlated. Because time-course studies are difficult to perform with transient transfection with the 120-kDa protein, are corre...
Regulation of TGA1a

Fig. 7. Xenobiotic stress induces transient changes in the rate of transcription of an as-1-regulated target gene of TGA1a. Results of nuclear run-on assays of de novo transcription from GNT35 and TGA1a genes are shown. Tobacco suspension cells were treated with 100 μM 2,4-D for 0, 0.5, 2, 4, and 8 h to induce xenobiotic stress, and nuclei were then isolated and used to generate labeled nascent transcripts as hybridization probes. The amount of labeled RNA (in cpm) that hybridized to Nytran-bound GNT35 and TGA1a cDNAs is shown. Due to its constitutive and xenobiotic stress-insensitive expression, transcription of TGA1a served as an internal control for differences in RNA recovery and labeling between samples.

TGA1a—One means by which xenobiotic stress might affect the activity of TGA1a is by inhibiting its ability to bind as-1. However, gel-shift experiments with nuclear extracts from mock- or 2,4-D-treated cells showed comparable amounts of a nuclear as-1 binding activity, termed “ASF-1” (data not shown). ASF-1 from these suspension cells is derived from the contributions of a number of different TGA factors (e.g. TGA2.1 and TGA2.2) that are more abundant than TGA1a (23). To therefore determine the specific contribution of the comparatively small amount of TGA1a to this activity, we used a sequential enrichment procedure involving DNA-affinity chromatography and anti-TGA1a immunoprecipitation (Fig. 8a). Results indicate that TGA1a from mock-treated cells failed to bind immobilized as-1 DNA (Fig. 8b, lane 4), unlike the activity of TGA1a from 2,4-D-treated cells (Fig. 8b, lane 10). Based on results with a mutant as-1 sequence, TGA1a binding was specific for as-1 (Fig. 8b, lanes 6 and 12). As expected, control reactions with preimmune sera did not recover detectable amounts of TGA1a from either of these extracts.

Interestingly, crude input fractions from mock-treated cells revealed the presence of a 120-kDa nuclear protein bound to TGA1a (Fig. 8b, lane 2), whereas TGA1a from the input nuclear fraction from 2,4-D-treated cells was not associated with this protein (Fig. 8b, lane 8). Based on the relative amounts of TGA1a in lanes 8 and 10 of Fig. 8b, we estimate that nearly all of the TGA1a present in the input fraction was recovered using as-1 DNA-affinity chromatography. The above-mentioned findings contrast sharply with the apparent inability of the TGA1a-120-kDa protein complex to bind as-1 (Fig. 8b, lane 4).

**DISCUSSION**

Plant transcription involving as-1-type cis-elements and their cognate TGA factors is activated by diverse cues including defense hormones, wounding, and xenobiotic stress. Of the known TGA factors, tobacco TGA1a is among the best characterized with regard to its contribution to transcription. TGA1a promotes as-1-dependent transcription in vitro (24) and as a transiently transfected chimeric factor in response to xenobiotic stress (12). In tobacco seedlings, TGA1a is preferentially coexpressed in root tip meristem cells along with several as-1-regulated genes, including GNT35, whose activities are further enhanced by xenobiotic stress (11). Gain of function studies with a heterologous system involving the yeast Gal4-binding DNA-affinity chromatography. The above-mentioned findings allowed us to identify separate contributions made by dimer stabilization and repression activities of the CT domain to as-1-dependent transcription. Enhanced trans-activity by TGA1a-CREBΔCT was shown to be largely due to the absence of the inhibitory CT domain and not due to the presence of the leucine zipper of CREB, as evidenced by the relatively poor trans-activity of the TGA1a-CREB control. In addition, immu-

![Fig. 8](image-url) Changes in as-1 binding activity of nuclear TGA1a and its association with a 120-kDa protein. a, flow diagram of the experiment. b, labeled nuclear proteins from tobacco suspension cells treated with 0.1% ethanol carrier solvent (Mock) or 100 μM 2,4-D (Xenobiotic stress) were analyzed directly as described below (input) or were incubated with wild-type (as-1) or mutant (mtas-1) immobilized oligonucleotides, washed, and then extracted from the DNA with radio-immune precipitation buffer (eluate). Input and eluate protein fractions were incubated with preimmune (P) or anti-TGA1a immune (I) sera, collected on Gammabind (protein G) Plus Sepharose resin, fractionated by SDS-PAGE, and detected by fluorography. The solid arrowhead indicates endogenous TGA1a, and the open arrowhead indicates the expected position of the 120-kDa protein. The apparent molecular masses of prestained protein markers are shown in kDa.
noprecipitation assays indicated that the enhanced activity of TGA1a-CREBACT was not due to significant comparative differences in its steady-state concentration, a finding that is further supported by our observations that further increasing expression of TGA1a or TGA1a-CREB had little additional stimulatory effect on transcription. We conclude from these data that the CT domain represses the basal activity of TGA1a.

During these studies, we observed that a 120-kDa protein bound TGA1a and that this interaction is responsive to xenobiotic stress. Evidence from several lines of investigation suggests that this TGA1a-binding protein may be a putative corepressor. First, its association with TGA1a was transiently affected by xenobiotic stress to a degree that was inversely correlated with the rate of expression of a TGA1a target gene, suggesting an inhibitory function. Second, binding by this 120-kDa protein to TGA1a specifically involved the regulatory CT domain of this factor, which mediates transcription in a heterologous system in response to xenobiotic stress. Deleting this domain from TGA1a abolished both its association with the 120-kDa protein and its ability to repress basal transcription activity, thus providing support for the notion that this TGA1a-binding protein has potential inhibitory activity.

In addition to the TGA1a-binding protein identified here, several other plant proteins have been shown to bind TGA factors. A ~26-kDa Arabidopsis protein termed OBPI associates with a subset of Arabidopsis TGA factors to facilitate their as-1 binding activity. OBPI1 itself is a DNA-binding factor and binds to AAGG motifs in as-1-regulated promoters (25, 26). A second Arabidopsis protein of ~66 kDa termed NRP1 (or NIM1) is a positive regulator of the as-1-regulated PR1 gene in response to pathogens and plant defense cues, such as salicylic acid. Like OBPI1, the NRP1 protein binds to a subset of TGA factors (27–29). Mutations in NRP1 that disrupt this binding activity also impair salicylic acid-induced expression of PR1, thus linking NRP1 activity to as-1. Intriguingly, Zhang et al. (27) have suggested a model whereby NRP1 potentiates transcription by reversing the binding of a repressor to one or more TGA factors. The apparent mass of the 120-kDa protein and its proposed mechanism of action appear to distinguish it from either OBPI1 or NRP1.

Results from this study indicate that TGA1a belongs to a subset of bZIP transcription factors whose activities are regulated through a mechanism involving stimulus-reversible repression. In the bZIP factor ATF-2, intramolecular binding under basal conditions occurs between the activation and bZIP domains and inhibits this factor’s DNA binding and trans-activation potential (30). In vitro, these inhibitory interactions in ATF-2 are alleviated by the coactivator CBP (31). A different type of regulatory mechanism occurs with CCAAT/enhancer-binding protein, a bZIP factor that negatively regulates its trans-activation potential via intramolecular interactions between activation and repressor domains through a cellular repressor protein (32). Transcription factors of the estrogen hormone receptor class represent yet another type of repression (33). When bound in the nucleus by the 90-kDa heat shock protein (hsp90), the estrogen receptor is unable to bind its cognate cis-element. Estrogens reverse this effect by inducing a conformational change in the receptor, thus promoting its release of hsp90 and subsequent binding to DNA.

Thus, how might xenobiotic stress affect the activity of TGA1a? One likely mechanism suggested here involves stimulus-induced changes in as-1 binding by TGA1a, perhaps through its stimulus-reversible association with a putative corepressor protein. Despite the fact that the 120-kDa protein bound to TGA1a in nuclear extracts from mock-treated cells, we were unable to detect this complex by gel-shift binding assay, suggesting that this complex may not bind DNA. This view is directly supported by evidence from an alternative TGA1a detection assay involving sequential DNA-affinity chromatography and immuno precipitation. By this approach, xenobiotic stress was found to potentiate the as-1-binding activity of TGA1a, presumably by inducing a change in this factor’s association with a 120-kDa protein. However, knowledge of the proportion of cellular TGA1a bound by this protein will be necessary before definitive conclusions regarding the regulatory contribution of this interaction to as-1 binding by TGA1a can be made.

Based on present and previous findings, we propose the following working model to explain how TGA1a affects as-1-dependent transcription. In the absence of xenobiotic stress, as-1 binding and trans-activation functions of TGA1a are inhibited. TGA1a’s loss of basal transcription activity occurs through this factor’s inhibitory CT domain, by a mechanism that may involve its stimulus-reversible interaction with a putative corepressor protein. In response to xenobiotic stress, TGA1a trans-activation and as-1 binding activities are enhanced. These changes occur in parallel with the release of the putative corepressor protein. Longer exposure to xenobiotic stress promotes reassociation of TGA1a with this protein and a concomitant decline in as-1-dependent transcription.

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