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Binding site requirements and differential representation of TGA factors in nuclear ASF-1 activity

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ABSTRACT

Activating sequence factor 1 (ASF-1) is a nuclear DNA-binding activity that is found in monocots and dicots. It interacts with several TGACG-containing elements that have been characterized from viral and T-DNA genes, the prototypes of which are the *as-1* element of the CaMV 35S promoter and the *ocs* element from the octopine synthase promoter. This class of *cis*-acting elements can respond to auxin and salicylic acid treatments. Consistent with these observations, we have shown that ASF-1 can interact with promoter elements of an auxin-inducible tobacco gene *GNT35*, encoding a glutathione S-transferase. Characterization of the nuclear factors that make up ASF-1 activity *in vivo* will be an important step toward understanding this induction phenomenon. The TGA family of basic-leucine-zipper (bZIP) proteins are good candidates for the ASF-1 nuclear factor. However, there may be as many as seven distinct TGA genes in Arabidopsis, five of which have now been reported. In this study, we expressed the cDNAs that encode four of these five Arabidopsis TGA factors *in vitro* and compared their DNA-binding behavior using two types of TGACG-containing elements. With specific antisera prepared against three of the five known Arabidopsis TGA factors, we also investigated the relative abundance of these three proteins within the ASF-1 activities of root and leaf nuclear extracts. Our results indicate that these TGA factors bind to DNA with different degrees of cooperativity and their relative affinity toward *as-1* also can differ significantly. The results of a supershift assay suggested that only one of the three TGA factors represented a significant component of nuclear ASF-1 activity. Arabidopsis TGA2 comprises ~33 and 50% of the ASF-1 activity detected in root and leaf nuclear extracts respectively. These results suggest that each member of the TGA factor family may be differentially regulated and that they may play different roles by virtue of their distinct DNA-binding characteristics. Furthermore, since transcripts for each of these factors can be detected in various plant tissues, post-transcriptional regulation may play an important part in determining their contribution to nuclear ASF-1 in a given cell type.

INTRODUCTION

Transcription factors play important roles in controlling the growth and development of living organisms. A good example is the MADS-box gene family that encodes transcription factors with a highly conserved DNA-binding domain. Several MADS-box factor-encoding genes correspond to homeotic genes involved in organ identity specification in the flowers of dicots (1). Another conserved family of plant transcription factors containing homeodomains that are homologous to the maize KNOTTED-1 gene product appears likely to regulate leaf morphogenesis (2). Like these proteins, many transcription factors exist as families with a significant number of members, each with very similar DNA-binding domains. Two important issues emerging from these observations are the features that distinguish one particular factor from others in the same family and the expression pattern of each individual gene product. Since these factors are likely to exert their influence on gene expression through sequence-specific interactions, modulation of a distinct subset of genes may result when these related factors are present in a particular combination. This prediction is illustrated by the observation that the combinatorial expression of several different MADS-box factors can result in specific organ identities during floral development (3). The implication is that each transcription factor family member contains a 'specificity' element allowing it to activate a distinct set of genes. Thus, subtle differences in DNA-binding activity and differential interactions with other proteins (e.g. protein kinases, coactivators, etc) are important determinants that may distinguish transcription factors with similar DNA-binding domains. In addition to this complexity, preferential expression of the various family members in distinct cell types and the modulation of their translocation into the nucleus, can further regulate the site of action for these proteins. Regulated nuclear transport was recently proposed for the G-box binding factor (GBF) in parsley cells (4) while differential expression of transcription factors is a well-established mode of regulation for these proteins in many systems including plants.

The plant *trans*-acting factor known as activating sequence factor 1 (ASF-1) was originally detected as a sequence-specific

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DNA-binding activity that interacts with the –75 region of the CaMV 35S promoter (5). This region of the 35S promoter contains two tandem TGACG motifs and is known to interact with upstream elements to activate transcription (6,7). Nucleotides at position –82 to –62 of the CaMV 35S promoter have thus been designated as activating sequence 1 (*as-1*) and interaction of this binding site with ASF-1 has been characterized extensively (8). We and others have shown that ASF-1 interacts with several other *cis*-acting elements from T-DNA genes and plant viral promoters. This has been shown using extracts prepared from monocots and dicots (9–12), thus indicating that ASF-1 is a highly conserved DNA-binding activity in higher plants. Recently, we and several other laboratories have shown that the transcription activity of *as-1* and other ASF-1 binding *cis*-elements are inducible by auxins and salicylic acid (13–16). Consistent with these observations, we have found that at least one auxin-responsive tobacco genes contains ASF-1 binding sites in its promoter region (13). This particular gene, called *GNT35*, encodes a new class of plant glutathione S-transferase (17). Recently Kim *et al.* (16) found, and we confirmed (Chengbin Xiang and E.L., unpublished data), that methyl-jasmonate is also a good inducer of *as-1* activity in transgenic plants and in transgenic tobacco cell cultures. We are thus interested in elucidating the signal transduction pathway(s) by which three different phytohormones can activate a common *cis*-element.

To study the molecular mechanism of the *as-1* promoter element, we and others have isolated cDNA clones from various plant species that encode DNA-binding proteins with similar sequence specificity to ASF-1. The first of these related cDNA clones was isolated from tobacco and was named TGA1a, since the relationship between the encoded protein and nuclear ASF-1 activity was unclear (18). TGA1a contains a basic-leucine-zipper (bZIP) motif found in mammalian factors that interact with similar TGACG motifs, such as the cAMP responsive element binding protein (CREB). Subsequently, a number of cDNA clones encoding proteins homologous to TGA1a were reported from monocots (12,19) and dicots (9,20–25). The early studies with tobacco indicated that TGA1a may belong to a multigene family containing at least two to four highly homologous genes (18). We have focused our research efforts in the past several years on the characterization of TGA1a homologues from Arabidopsis, with the expectation that there may be fewer genetic redundancies in this smaller genome. However, the results of low stringency Southern blot analyses indicated that there may be as many as seven distinct TGA1a related genes in Arabidopsis, designated as the TGA family (25). Five of these Arabidopsis TGA gene family members have been reported (20,21,23,25). The existence of multiple TGA1a-related genes suggests that nuclear ASF-1 activity may be comprised of distinct gene products from different members of the TGA family. In this paper, we carried out *in vitro* DNA binding studies with four different TGA factors from Arabidopsis to determine if they possess different DNA binding sequence requirements as well as cooperativity in DNA binding. In addition, the contribution of three different TGA factors to nuclear ASF-1 activity in root and leaf was examined using specific antisera. Our results indicate that the TGA factors which we compared have distinct DNA binding characteristics and that their representation in nuclear ASF-1 is not correlated with the expression of their transcripts in leaf and root tissues. The latter result suggests that post-transcriptional mechanisms may determine the accumulation of active TGA factors in the nucleus.

MATERIALS AND METHODS

PCR cloning and *in vitro* expression of Arabidopsis TGA1, TGA2, TGA3 and TGA5

Amplification of TGA and G- box binding factor by polymerase chain reaction (PCR). The coding regions for GBF1, TGA1, TGA2 and TGA3 were amplified from phage DNA prepared from an Arabidopsis cDNA library, as described previously (25,30). For TGA5, the following primers were used: EL424 (5′-CGGATC-CATGAGAACATCAGTCTCAAC) and EL426 (5′-CGTCGAC-TCAAGATCTCTCTCTTGGTCTGGCAA). cDNA made from polyA$^+$ RNA purified from 3 day old Arabidopsis seedlings were used as templates. The PCR cycle used was: 94°C, 1 min; 52°C, 1 min; 72°C, 1 min for 35 cycles. After amplification, the products were purified by agarose gel electrophoresis and then reamplified. After subcloning into the pGEM-T vector (Promega), each of these sequences were completely sequenced to confirm the absence of any mutation. For *in vitro* transcription/translation, each of the inserts were subcloned into the pET23a vector (Novagen) and the TNT coupled transcription/translation system (Promega Co.) was used to produce ^{35}S-labelled proteins (30).

Construction of N-terminal fusion constructs and antisera preparation

Amplification of the N-terminal regions of TGA1, TGA2 and TGA3. The N-terminal 86 amino acids of TGA1 was amplified by PCR from the cDNA clone using the primers: EL317 (5′-CGG-ATCCATGAATTCGACATCGACACA) and EL384 (5′-CGTA-AGCTTCTATTGTATCTTATGGGGATG). The N-terminus of TGA2, which corresponds to the first 48 amino acids, were amplified using two primers (EL318: 5′-CGGATCCATGGCTG-ATACCAGTCCGAG; EL391: 5′-CGTCGACTCAAGATCTA-AGAGTCTTTTGATCC) while for TGA3, the primers used were: EL319 (5′-CGGATCCATGGAGATGATGAGCTCTTC) and EL392 (5′-CGTCGACTCAAGATCTCATCTTATCATTG-ATCCG) in order to amplify the region encoding the first 99 amino acid residues. Each of these were subcloned in the pGEM-T vector and sequenced completely to verify the absence of mutations during amplification. To aid in their subsequent manipulations, each set of these primers introduced a *Bam*HI site in frame with the first ATG of the cDNA clones. In the 3′ ends of these fragments, a *Bgl*II site followed by a stop codon and a *Sal*I site were added. Since the TGA2 N-terminal region is rather short compared with the other two, we multimerized it by ligating a *Bam*HI/*Bgl*II fragment from this clone into the *Bgl*II site at the 3′ end of the TGA2 insert. This resulted in a fragment encoding 96 amino acids rather than 48 residues. Each of the N-terminal regions was then subcloned into the pMAL-c2 vector (New England BioLabs) at the *Bam*HI/*Sal*I sites and expression of the fusion proteins induced by IPTG.

Raising polyclonal antibodies directed against the fusion proteins. Fusion proteins were produced in 1l cultures of BL21(DE3)pLys5 cells. Preparative gel electrophoresis was performed to purify the fusion proteins from the crude bacterial extract using the model 491 PrepCell (BioRad) according to the maunfacturer's instructions. About 150 mg of each fusion protein was used for rabbit immunization. Four weeks after the first immunization, a second dose of 150 mg of each fusion protein was injected into the

corresponding rabbit. Antisera were collected 10 weeks after the first immunization.

Gel mobility shift assays, extract preparations and protein gel blot analysis

DNA–protein interaction was assayed by gel mobility shift assay essentially as described previously (25).

Nuclear extracts from Arabidopsis tissues were prepared as described previously (25). The crude bacterial extracts were used in western blot analysis with the antisera. The bacterial proteins were first separated by electrophoresis on a 10% SDS–polyacrylamide gel and then transferred onto an Immobilon-P transfer membrane (Millipore). The membrane was blocked in TBS-T (20 mM Tris–HCl pH 7.4, 150 mM NaCl and 0.5% Tween 20) containing 5% non-fat dried milk overnight with shaking. It was washed twice for 5 min in TBS-T and then incubated in the corresponding antiserum diluted in 1% BSA/TBS-T for 1 h at room temperature (1:1500 dilution for the treated TGA1 and TGA2 antisera, 1:500 dilution for the treated TGA3 antiserum). The membranes were washed in TBS-T six times for 10 min. They were then incubated with horseradish peroxidase (HRP) conjugated secondary antibody at a dilution of 1:3000 for 1 h at room temperature and then washed six times for 10 min in TBS-T. It was finally treated with the Renaissance (Du Pont) chemiluminescent detection reagents according to the manufacturer's protocols and then exposed to autoradiography film (Kodak).

RESULTS

In vitro production of Arabidopsis TGA factors

As noted by Miao *et al.* (25) and Foster *et al.* (26), TGA factors are conserved bZIP proteins found in both monocots and dicots. Aside from significant conservation of the amino acid sequences throughout the bZIP and C-terminal regions that have been noted previously (25), a 19-residue stretch in the basic domain region is found to be invariant among the 13 known sequences. In contrast, a distinct 14-residue stretch in the same region is found to be conserved amongst the G-box binding factors (GBF), another conserved family of plant bZIP proteins (26). Since the basic domain of bZIP proteins is known to be involved in sequence recognition (27), we would expect that these different TGA gene products are likely to have similar DNA binding sequence requirements. Certain residues in and around the leucine zipper region of these TGA factors are also highly conserved. Since each of the reported TGA factors studied to date has been shown to form stable homodimers, the conservation of amino acid residues in the leucine zipper region suggests that these proteins are likely to form heterodimers with each other as well (28). In addition, the C-terminus of tobacco TGA1a has been demonstrated to stabilize dimer formation (29). Thus, the observed sequence conservation between the C-termini of these different TGA factors suggests that these proteins can form stable heterodimers (25). This expectation was demonstrated recently by showing that a synthetic dominant negative mutant of tobacco TGA1a, in which the basic domain was deleted, suppressed DNA-binding when co-expressed with the Arabidopsis TGA factors *in vitro* (30). Although these similarities suggest that the different TGA factors are likely to share common characteristics, it remains possible that each may behave in a distinct manner with respect to their optimal binding site sequences and protein–protein interaction properties. The cloning of five different TGA factors from Arabidopsis also suggests that they may have different functional and/or regulatory properties. To address these questions, we expressed four of the five reported Arabidopsis TGA factors by *in vitro* transcription and translation using rabbit recticulocyte lysates (Fig. 1). The products were radiolabeled by [^{35}S]methionine incorporation in order to facilitate the estimation of their relative quantities. Using comparable amounts of these radiolabeled proteins, we compared their behavior in binding to the *as-1* site by gel mobility shift assay (Fig. 2). Each of the two TGACG motifs in the *as-1* site can interact with one ASF-1 factor, thus generating a slower mobility DNA–protein complex when both sites are occupied (10,31). The faster moving complex (the lower band) represents occupation of either one of the two TGACG motifs by one dimer of TGA factors. With similar amounts of TGA1, TGA2, TGA3 and TGA5 separately produced *in vitro*, we carried out protein titrations to compare their relative affinity toward the *as-1* site and detect evidence for cooperative interactions between factors bound to adjacent sites. The data from the titration experiments are shown in Figure 2a. The amounts of probe bound in the upper and lower bands were quantitated in relation to the total amount of *as-1* probe added in each assay (Fig. 2b). The apparent affinity of TGA1 for *as-1* is ~2-fold lower than the other three TGA factors. Since interaction between neighboring factors bound to adjacent TGACG sites may increase the stability of these bound proteins on the *as-1* site, rapid appearance of the upper band, in which both TGACG motifs are presumed to be occupied (10,31), is a good indicator of cooperative interactions. As the quantitative data in Figure 2b illustrates, TGA1 and TGA3 showed no significant cooperation in binding to the two tandem TGACG motifs in *as-1*. Thus, for both TGA1 and TGA3, very little of the upper complex was observed even when 20% of the total probe was bound. In contrast, the upper complex was readily detected even at comparatively low amounts of added TGA2 and TGA5. The similar behavior of TGA2 and TGA5 is not surprising since they share a high degree of overall homology at the amino acid sequence level (>80%). However, the apparent affinity of TGA5 for *as-1* appeared slightly higher than TGA2. It should be noted that the different TGA factors may have distinct preference for

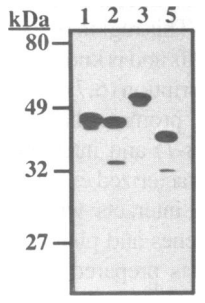

Figure 1. *In vitro* production of Arabidopsis TGA factors. The complete cDNA sequences of Arabidopsis TGA1, TGA2, TGA3 and TGA5 were expressed under the control of the T7 promoter using the TNT recticulocyte lysate *in vitro* transcription-translation system (Promega). The amounts of templates for each TGA factor were adjusted to produce similar amounts of proteins as quantitated by the [^{35}S]methionine incorporation estimated from autoradiography. Each lane in the SDS–PAGE gel shown contains 1 µl of each programmed lysate reaction mixture. The positions of the pre-stained molecular mass markers (in kDa) are indicated on the left. Designations of the lanes are: 1, TGA1; 2, TGA2; 3, TGA3; 5, TGA5.

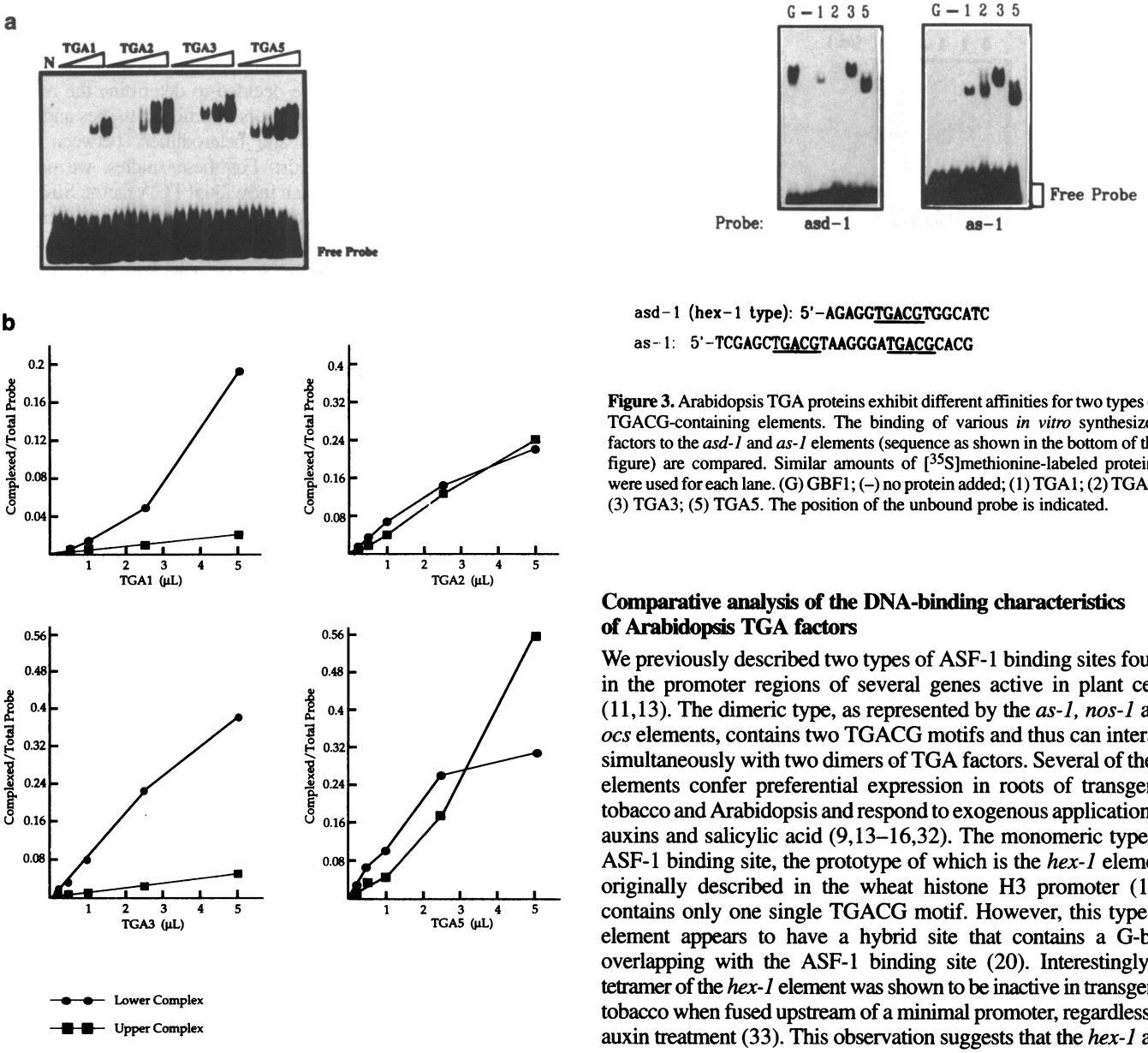

Figure 2. Titration of the *as-1* element with four Arabidopsis TGA factors. (**a**) Gel mobility shift assay of TGA factor binding to the *as-1* site. A probe containing a single *as-1* element consisting of two TGACG motifs was labeled with ^{32}P and used for titration analysis with TGA1, TGA2, TGA3 and TGA5. The leftmost lane (N) contains the probe alone in the reaction mixture. For TGA1, 0.5, 1, 2.5 and 5 μl of lysate mixture was used. For the other three TGA factors, 0.2, 0.5, 1, 2.5 and 5 μl of lysate mixtures were added as indicated. The position of the free, unbound probe is indicated. (**b**) Measurement of the amount of *as-1*/TGA complexes. A PhosphorImager (Molecular Dynamics) was used to estimate the amount of complexed *as-1* probe in the upper and lower bands. The values are normalized to the amount of probe added; a value of 1.0 is equivalent to the total labeled *as-1* probe added. Values for the lower and upper complexes are indicated by closed circles and squares, respectively. Note different scale was used for the data with TGA1.

one of the two TGACG motifs within the *as-1* site. Thus, the faster mobility complex in each case may represent occupation of either of the two TGACG-containing sequences.

Figure 3. Arabidopsis TGA proteins exhibit different affinities for two types of TGACG-containing elements. The binding of various *in vitro* synthesized factors to the *asd-1* and *as-1* elements (sequence as shown in the bottom of the figure) are compared. Similar amounts of [^{35}S]methionine-labeled proteins were used for each lane. (G) GBF1; (–) no protein added; (1) TGA1; (2) TGA2; (3) TGA3; (5) TGA5. The position of the unbound probe is indicated.

Comparative analysis of the DNA-binding characteristics of Arabidopsis TGA factors

We previously described two types of ASF-1 binding sites found in the promoter regions of several genes active in plant cells (11,13). The dimeric type, as represented by the *as-1, nos-1* and *ocs* elements, contains two TGACG motifs and thus can interact simultaneously with two dimers of TGA factors. Several of these elements confer preferential expression in roots of transgenic tobacco and Arabidopsis and respond to exogenous application of auxins and salicylic acid (9,13–16,32). The monomeric type of ASF-1 binding site, the prototype of which is the *hex-1* element originally described in the wheat histone H3 promoter (12), contains only one single TGACG motif. However, this type of element appears to have a hybrid site that contains a G-box overlapping with the ASF-1 binding site (20). Interestingly, a tetramer of the *hex-1* element was shown to be inactive in transgenic tobacco when fused upstream of a minimal promoter, regardless of auxin treatment (33). This observation suggests that the *hex-1* and *as-1* type elements interact differently with TGA factors, in addition to the possibility that other nuclear factors such as TGA1b and GBF can interact with the *hex-1* site and thus alter its properties in transactivation (11,20). We compared the binding of each of the four TGA factors to a *hex-1* like element, *asd-1* (13), found in the promoter of the auxin-inducible *dbp* gene from Arabidopsis (34). As a control, we also expressed GBF1 *in vitro* and examined its binding activity toward *asd-1* and *as-1* (Fig. 3). We found that, as expected from the previous work of Schindler *et al.* (20), GBF1 preferentially bound to *asd-1*, the *hex-1* type element, and did not bind significantly to the *as-1* site. In contrast, TGA1 preferentially interacted with the dimeric site of *as-1* with lower binding to the *asd-1* site. Interestingly, TGA2 showed little detectable binding to a probe with a single *asd-1* site while strong interaction was observed with the *as-1* element. Little difference was observed in the binding of TGA3 and TGA5 to the two different types of binding sites (Fig. 3, lanes 3 and 5). These results show that although each of these four TGA factors can interact with the *as-1* site and share a highly homologous bZIP domain, they have distinct preferences for DNA recognition sites.

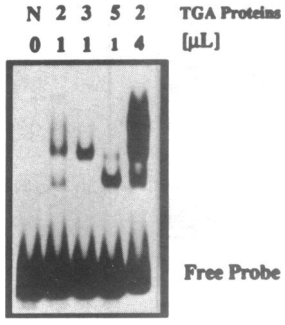

Figure 4. TGA2 can bind to a tetramer of *hex-1*. The binding of TGA2 to a probe consisting of a tandem tetramer of the *hex-1* sequence (18) was compared with TGA3 and TGA5. Designation of the lanes are as in Figure 2 except the amount of lysate mixtures used in each sample is also indicated.

Binding site number dependency of TGA2 is distinct from the other Arabidopsis TGA factors

The inability of TGA2 to interact with the *hex-1* type element may be due to two possibilities: (i) this factor may have a different sequence requirement from the other TGA factors examined and cannot bind to the *asd-1* site; (ii) TGA2 may require two or more TGACG motifs in order to bind efficiently. To test these alternatives, we compared the binding of TGA2, TGA3 and TGA5 to a tetramer of the *hex-1* element. If TGA2 cannot recognize the *hex-1* type element at all, then multimerization of this element would have no effect on binding by TGA2. However, if a simple requirement of binding site number determines the apparent affinity of TGA2 to the TGACG element, we would then observe increased binding with the tetramer probe. Figure 4 shows that TGA2 can bind to a *hex-1* tetramer as well as the other two TGA factors, thus showing that the reason for its inability to interact with the *asd-1* probe (Fig. 3) is likely the absence of multiple binding sites on the same probe. As seen with the *as-1* site (Fig. 2), binding of TGA2 to the *hex-1* tetramer probe appears to be cooperative, as suggested by the appearance of the upper band indicative of multiple proteins binding to the same probe at relatively low concentrations of added TGA2. Since there are four possible binding sites on the *hex-1* tetramer probe, we would predict four distinct DNA–protein complexes with different mobilities in the gel shift assay. These complexes should represent the different degrees of saturation of the four possible binding sites on the added probe. Indeed, as we increased the amount of TGA2 we observed additional DNA–protein complexes with slower mobilities (Fig. 4, lane 5). It is interesting to note that we can detect the presence of a fast mobility TGA2/DNA complex that is indicative of the occupation of a single site when a probe with multiple sites was used. Since TGA2 binds very poorly to a probe with a single site (Fig. 3), this observation indicates that the on-rate of TGA2/DNA interaction is the parameter that depended on binding site number.

Generation of specific antisera that recognize three different Arabidopsis TGA factors

Previous Southern analyses indicated that there may be as many as seven distinct Arabidopsis genes encoding homologous TGA factors with a conserved bZIP domain that can interact with the *as-1* type elements (25). Each of these proteins is a potential candidate for components of the nuclear ASF-1 factor that mediate the activity of this class of *cis*-elements. To understand the role of each of these TGA factors, we decided to determine the relative concentrations of each of these factors in different tissues and what combinations of homodimers and heterodimers between these different TGA factors may exist. For these studies, we needed specific probes to quantitate each individual TGA factor. Since the TGA factors contain a highly conserved DNA-binding domain and a homologous C-terminus, polyclonal antisera raised against a complete TGA gene product might cross-react with some or all of the other TGA factors. On the other hand, generating and screening for monoclonal antibodies specific for each TGA factor would be laborious and expensive. To circumvent these problems, we took advantage of the observation that the N-termini of TGA1, TGA2 and TGA3 are divergent in amino acid sequence (25) and generated fusion proteins of these regions. Thus, cDNA sequences encoding the N-terminal portions of these three TGA factors were amplified by PCR and the desired product subcloned into a bacterial expression vector as translational fusions to the *E.coli* maltose binding protein (MBP). These fusion proteins were expressed in bacteria (Fig. 5) and after their purification, they were injected into rabbits in order to raise polyclonal antibodies against these portions of the three TGA factors. To deplete the resulting antisera from antibodies that recognize MBP or other bacterial proteins, we incubated them with Sepharose beads cross-linked with total extracts from an *E.coli* strain overexpressing MBP. The antisera were then used in protein gel blot analyses with extracts from bacteria expressing TGA1, TGA2 and TGA3 to determine their specificities (Fig. 5). We found it difficult to obtain an *E.coli* strain that overexpressed full-length TGA2. The growth of the bacteria containing this expression vector was severely arrested upon IPTG induction, which accounts for the lower amount of total proteins observed for this sample in the coomassie blue-stained gel of Figure 5. Nevertheless, we found that the three antisera generated from the fusion proteins recognized the respective full-length TGA factors from which the N-termini were obtained and exhibited no detectable cross-reactivity with the other two factors. The specificity of these antisera was further established by a supershift assay in which each TGA factor produced *in vitro* (see Fig. 1) was detected as a DNA–protein complex. Interactions with the respective antisera were revealed by a further reduction of the mobility of this complex. As Figure 6a shows, each of the *as-1*/TGA factor complexes was supershifted with only one antiserum. Incubation with preimmune sera gave no detectable supershifts. The quantitative conversion of each *as-1*/protein complex into a supershifted complex by a specific antiserum suggests that these slower complexes in the gel shift assay are not artefacts of antisera addition. In addition, the data in Figure 6a demonstrates that each of the three TGA factors expressed in bacteria can be detected by the supershift assay.

During the course of this work, the sequence of TGA4 and TGA5 were reported (23). Since TGA5 has significant homology to TGA2 throughout its sequence, including the N-terminal region, we wanted to determine whether anti-TGA2 serum could cross-react with TGA5. Figure 6b shows that no detectable cross-reaction to TGA5 was observed in the supershift assay. This was further confirmed by protein gel blot analysis using a recombinant *E.coli* strain that overexpresses TGA5. No cross-reactivity with anti-TGA2 serum was observed (E.L., data not shown). Interestingly, in contrast to TGA2, full-length TGA5 could be easily overpro-

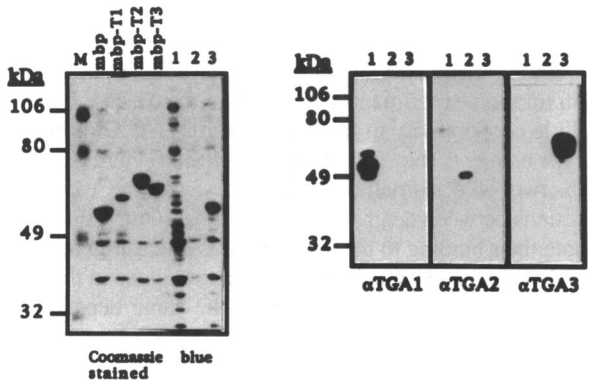

Figure 5. Production of gene-specific antisera against the N-termini of TGA1, TGA2 and TGA3. (Left panel) The fusion proteins between the N-termini of TGA1, TGA2 and TGA3 with maltose binding protein (MBP) were constructed as described in Materials and Methods. Bacterial extracts overproducing these fusion proteins were run in an SDS–PAGE gel and stained with coomassie blue. M, BioRad pre-stained molecular mass markers; mbp, original cloning vector with MBP only; mbp-T1, MBP-TGA1 fusion; mbp-T2, MBP-TGA2 fusion; mbp-T3, MBP-TGA3 fusion. Total proteins from *E.coli* strains overexpressing the full-length cDNA of TGA1 (1), TGA2 (2) and TGA3 (3) were also shown. (Right panel) Polyclonal antisera prepared from the three fusion proteins were used for protein gel blot analyses with bacterial extracts with TGA1 (1), TGA2 (2) and TGA3 (3). The identity of the antiserum used for the different blots is indicated on the bottom. Pre-immune sera from these rabbits did not exhibit reactivity with the recombinant TGA factors (results not shown). Apparent molecular masses are indicated to the left of the panels in kDa.

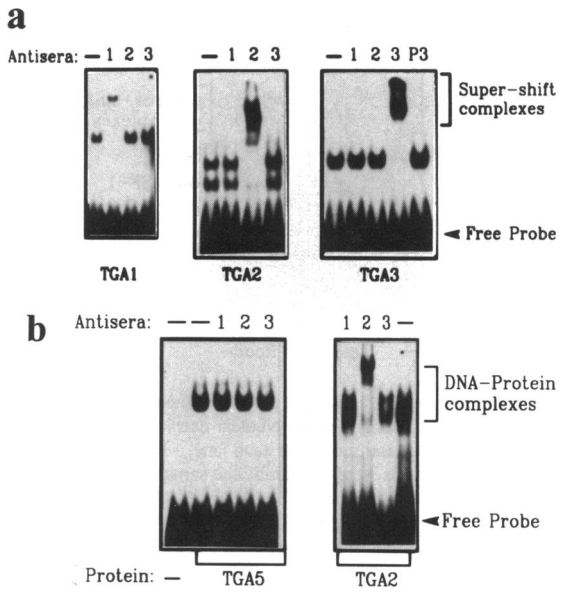

Figure 6. Specificity of the antisera toward individual TGA factors determined by the supershift assay. (**a**) Gel mobility shift assays were carried out with *in vitro* synthesized TGA1, TGA2, TGA3 factors (as indicated at the bottom of each panel) and addition of the different antisera is indicated on the top. (–) no antiserum added; (1) anti-TGA1; (2) anti-TGA2; (3) anti-TGA3; (P3) pre-immune serum for anti-TGA3. The probe used in this set of experiment was a tetramer of *as-1* (5). The position for the supershifted complexes is indicated on the right. (**b**) TGA5 is not recognized by antiserum generated against TGA2 N-terminus fusion protein. The supershift assay was performed essentially as in a except that a probe consisting of a single *as-1* site was used instead of the *as-1* tetramer. *In vitro* synthesized TGA5 and TGA2 were compared in their reactivity with antisera against each of the three TGA factors shown in Figure 5. Designations of the lanes are as in (a). For each supershift assay, 1 µl of a 1:10 (v/v) diluted antiserum stock was added to a 10 µl binding reaction from the start of the incubation along with the other components.

duced in the BL21/DE3 bacterial strain used for the production of recombinant proteins. Thus, even though the N-termini of TGA2 and TGA5 exhibited ~80% sequence identity, the antiserum generated against TGA2 was unable to cross-react with this closely related homologue. These results suggest that our approach may be useful for generating highly specific antisera for closely related members of a gene family. Our antisera should be useful for measuring the relative concentrations of TGA1, TGA2 and TGA3 in nuclear extracts of Arabidopsis. Since we were unable to detect the *in vitro* translated proteins on a western blot even when 5-fold more protein than that for gel shift assays were used (E.L., data not shown), the supershift assay appeared to be significantly more sensitive than the immunoblot assay.

Detection of TGA2 in root and leaf nuclear extracts of Arabidopsis

Using these specific antisera, we performed supershift assays with Arabidopsis leaf and root nuclear extracts (Fig. 7). The *as-1* probe was used to detect ASF-1 activity in both of these extracts and comparable levels of DNA–protein complexes were observed with similar amounts of nuclear proteins from these tissues. We failed to detect any evidence for interaction of anti-TGA1 and anti-TGA3 sera with nuclear ASF-1 from either leaf or root. With anti-TGA2 serum, however, we observed the formation of supershifted complexes when it was added to nuclear extracts and *as-1* probe. The amount of the detected ASF-1 complex with normal mobility was concomitantly decreased along with the appearance of the supershift complexes. We quantitated the relative amounts of these complexes using a PhosphorImager to estimate the contribution of TGA2 to leaf and root nuclear ASF-1 activity. About 50% of the leaf and 33% of the root nuclear ASF-1 activities apparently contain TGA2 in extracts prepared from Arabidopsis roots and rosette leaves. The amount of radioactive probe found in the supershifted complexes corresponded to the amount lost in the *as-1*/ASF-1 complex. The quantitative conversion of ASF-1 complex with normal gel shift mobility to the supershifted complex provides strong support for the identity of the observed complex and not due to an artefact derived from the antisera. It should be pointed out that these supershifted complexes may contain both TGA2 homodimers as well as heterodimers between TGA2 and other undefined partners, possibly other TGA factors. This may in part give rise to the apparent heterogeneity in the supershifted complexes observed, as shown by the multiple bands of different mobilities (Fig. 7).

DISCUSSION

Complexity of TGA factors and other bZIP proteins as transcription factor families

It is now recognized that many eukaryotic transcription factors do not function as independent units. In many cases, physical interaction between a transcription factor and other proteins plays an important role in modulating subcellular localization or functional properties (35,36). For bZIP proteins, this was demonstrated with classical examples such as the Jun and Fos oncoproteins where the heterodimer between these two proteins is apparently the functional species *in vivo* and homodimers of either Jun or Fos are weak transcriptional activators at best (37). For many bZIP transcription factors from animal and plant systems, distinct families of

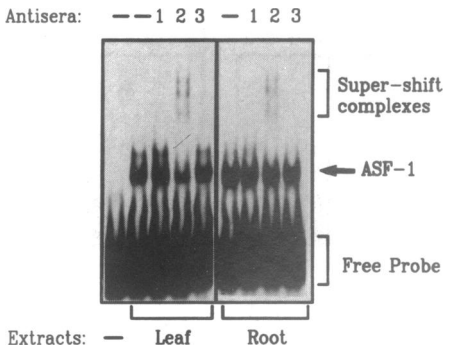

Figure 7. TGA2 comprises a significant portion of the nuclear ASF-1 activity in Arabidopsis rosette leaves and roots. Nuclear extracts were prepared from Arabidopsis rosette leaves and roots. For each lane, ~1 μg of nuclear extracts was added. Other conditions were essentially the same as those for Figure 6b. The positions of the free unbound probe, the *as-1*/ASF-1 complex and the supershifted complexes are indicated.

related proteins are found to contain highly conserved DNA-binding domains (26,30,38–40). One possible function for the existence of multiple factors containing similar DNA-binding and oligomerization domains is that one can generate a large repertoire of transcription factors with distinct properties by combinatorial pairing of a relatively small set of gene products (35). Implicit in this model is that there are likely specificity elements within the divergent regions of the transcription factor family members which may distinguish them from each other. Thus, each homodimer and heterodimer species will have distinct functional properties that optimize them for a specific role *in vivo*. This is well illustrated by the work of Chiu *et al.* (39) who showed that transactivation by different Jun homologues is dependent upon distinct promoter structures. Thus, JunB can activate promoters with multiple binding elements but is unable to stimulate transcription from those with a single site. c-*Jun* does not have such a requirement and activates transcription from both types of promoter. When coexpressed, JunB can apparently suppress the activity of c-*Jun*.

From the five members that have been cloned, the Arabidopsis TGA factor family has the potential to generate 15 different combinations of homodimers and heterodimers. Although each of these dimers would be expected to bind to similar TGACG motif-containing sites, the divergent regions found in each of these five distinct TGA gene products may confer different functional and regulatory properties to the various species of stable dimers. With this study, we have initiated a systematic comparison of the DNA-binding properties of several Arabidopsis TGA factors. Our eventual goal is to establish a comprehensive set of biochemical data regarding all the TGA factors from this higher plant species and to define their roles in plant development. Although the bZIP regions of the different TGA factors appear to be highly conserved, we show that they are likely to have distinct DNA-binding properties. Under our gel mobility shift assay conditions, each of the four Arabidopsis TGA factors can bind to the *as-1* probe and the electrophoretic mobilities of these complexes are inversely proportional to the relative sizes of these proteins (Figs 1 and 2a). Thus, the larger TGA proteins such as TGA3 gave rise to a complex with the lower mobility. It is interesting to note that the faster mobility of the TGA2/TGA5-containing complexes is reminiscent of the nuclear ASF-1 complex of Arabidopsis that we noted earlier (25). This suggested that the smaller members of the TGA protein family may be the main contributors to this DNA-binding activity. Comparison of the four TGA factors by protein titration experiments suggests that TGA1 and TGA3 bind with little cooperativity to the two tandem TGACG motifs in the *as-1* type elements (Fig. 2b). In contrast, the binding of TGA2 and TGA5, two of the smaller TGA factors, showed evidence for interactions between neighboring factors that may stabilize and/or facilitate their binding to the *as-1* site. It is interesting to note that although TGA2 and TGA5 are highly homologous in their amino acid sequences, TGA5 does not have the same dependence on multiple sites for efficient DNA binding. In this respect, TGA5 is more similar to the nuclear ASF-1 activity reported earlier (11).

Possible differential representation of TGA factors in nuclear ASF-1 activity

The finding of multiple TGA factor-encoding genes in a given plant species raised a complex question of which member may be contributing toward nuclear ASF-1 activity. Using specific antisera, we obtained results by supershift analyses with Arabidopsis nuclear extracts that suggested two conclusions: (i) TGA1 and TGA3 may not make a significant contribution to either leaf or root nuclear ASF-1 activity; and (ii) TGA2 comprises ~50 and 33% of the ASF-1 factor activity in leaf and root nuclei, respectively. It is particularly surprising to find that TGA1 and TGA3 were not detectable in root ASF-1 activity since both of these genes are expressed preferentially in the root tissues of Arabidopsis (20,25). From RNA gel blot analysis, we can detect expression of each of the five reported Arabidopsis TGA genes in leaves and roots (C. Xiang and Lam, unpublished results). Thus, the absence of TGA1 and TGA3 in nuclear ASF-1 activities suggests that post-transcriptional regulation may modulate either the translation of these transcripts, the nuclear entry of these proteins and/or their DNA-binding activities. Post-transcriptional regulation of TGA factors such as Arabidopsis TGA1 and TGA3 may be conserved in different plant species since we observed similar behavior for the well-characterized tobacco TGA1a. Thus, no evidence for the presence of TGA1a was found by supershift assays with tobacco leaf nuclear ASF-1 using an anti-TGA1a serum (E.L., data not shown). The finding that TGA2 is a significant contributor to nuclear ASF-1 is consistent with the observed similarity in gel shift mobilities of the *as-1*/TGA2 and *as-1*/ASF-1 complexes, as noted above. Since TGA2 only constitutes part of the total ASF-1 activity *in vivo*, the identity of the other participating proteins remains to be determined. Based on the similarity of the size and deduced amino acid sequences between TGA5 and TGA2, it is quite possible that TGA5 may be present as well. Specific antisera to TGA5 should allow us to test this in the future. At present, it is possible that the N-terminal region of TGA1 and TGA3 may be interacting with another protein, thus rendering the epitopes in this region of these factors inaccessible to our antisera. An example of such a protein may be GF14 which associates with the nuclear GBF complexes (42). However, we think this is unlikely since the addition of another protein to a TGA factor dimer should further retard its mobility in our gel shift assay. This would not be consistent with our observation that the nuclear ASF-1 activity is significantly faster in its mobility than TGA1 and TGA3 homodimers in this assay. Another possibility for our failure to detect TGA1 and TGA3 via the supershift assay may be due to protein modification(s) that resulted in inhibition of their interac-

tion with the antisera. For example, *in vivo* phosphorylation of these TGA factors may result in a conformational change that 'masked' the epitopes recognized by our antisera. Protein modifications may also involve proteolytic processing of the translated proteins that may have removed the particular epitopes. It is also possible that translation of the expressed transcripts for these genes is regulated. The maize bZIP transcriptional regulator Opaque-2 apparently undergoes this type of regulation (43). Alternatively, they may be expressed in the cytoplasm but are physically inhibited from entering the nucleus by interaction with other cellular proteins. This appears to be the case for parsley GBF (4) and the Arabidopsis COP1 protein involved in the suppression of photomorphogenic development (44). In both cases, light appears to regulate the subcellular localization process. We have tried to distinguish these different possible modes of regulation by carrying out western blot analyses with Arabidopsis nuclear extracts using the antisera directed against TGA1, TGA2 and TGA3. With up to 10 μg of nuclear proteins, we can barely detect TGA2 and have no success in detecting TGA1 and TGA3 at all with our antisera (data not shown). These observations are consistent with a higher concentration of TGA2 protein within the nuclear extracts as compared with TGA1 and TGA3. However, more quantitative immunoblot analyses in the future with these antisera and others directed against the remaining TGA factor family members of Arabidopsis will be necessary to provide a more complete description of the relative abundance of these related DNA-binding proteins. In any case, our current working model favors differential translation and/or post-translational nuclear entry as regulatory mechanisms that may modulate the abundance of individual TGA factors in the nucleus of a particular cell.

The functional roles of TGA1a, TGA1 and TGA3 remain enigmatic at present. When expressed *in vitro*, they behave as DNA-binding proteins with well-defined sequence specificities and in the case of TGA1a, a *bona fide* transcription activator. Nuclear entry of TGA1a is observed when it was overexpressed as a fusion protein with the bacterial reporter gene β-glucuronidase (45). Furthermore, TGA1a can activate *as-1* dependent transcription when microinjected into plant cells as a purified recombinant protein (46). These results, however, may be complicated by the possibility that the presence of a large amount of a particular TGA protein can overwhelm the regulatory pathways that normally control these factors. In any case, future studies using targeted mutagenesis of individual TGA gene family members (41) or construction of gene-specific dominant negative mutants (30) may allow us to ascertain the functional role for these conserved regulators.

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