

Auxin-Sensitive Elements from Promoters of Tobacco *GST* Genes and a Consensus *as-1*-Like Element Differ Only in Relative Strength¹

Bert J. van der Zaal*, Frans N. J. Droog, Frank J. Pieterse, and Paul J. J. Hooykaas

Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

We have investigated the *cis*-acting potential of several *as* elements (20-bp *as-1/ocs*-like sequences) in both yeast and plant cells. These TGACG[N₇]TGACG-resembling elements were surprisingly similar with respect to their ability to confer inducibility by auxins and related compounds to a heterologous TATA box in stably transformed plant cells. Both in plant cells and in yeast it was found that differences between *as* elements were of a quantitative nature. A strong element based on the consensus sequence for *as* elements conferred the highest level of gene expression. The rather aberrant *as* elements present in the promoters of auxin-inducible *gst* genes *Nt103* and *Nt114* of tobacco were much weaker *cis*-acting elements. The ability of an element to drive reporter gene expression was found to correlate with the extent to which proteins present in (nuclear) extracts of yeast and plant cells bound to it. The cloned transcription factor TGA1a was shown to be a very good candidate to be the factor that mediates the *in vivo* regulation of gene expression via *as* elements. The physiological significance of gene activation by active and inactive auxins is discussed.

The plant hormone auxin is involved in multiple processes regulating plant development such as cell elongation, cell division, and maintenance of apical dominance. To gain insight into auxin signal transduction, genes have been studied that are transcriptionally activated by auxins in systems reacting with cell elongation (Walker and Key, 1982; Hagen and Guilfoyle, 1984; Theologis et al., 1985; McClure and Guilfoyle, 1987; Yamamoto et al., 1992) or cell division (van der Zaal et al., 1987, 1991; Takahashi et al., 1989). AuxREs, which are indispensable for auxin-induced gene expression, are currently being defined (Ballas et al., 1993; Nagao et al., 1993; Li et al., 1994; Liu et al., 1994; Droog et al., 1995a). When *trans*-acting factors binding to these elements can be identified, it will be possible to trace other components of the signal transduction pathway.

So far at least two different AuxREs appear to exist (for a recent review, see Napier and Venis, 1995). The most widely conserved AuxRE present in genes isolated from elongating tissues appears to be a T/GGTCCCAT motif, as was demonstrated by Theologis's group (Ballas et al., 1993;

Oeller et al., 1993). Genes containing this motif are supposedly induced only by active auxins. Another type of AuxRE with a TGACG[N₇]TGACG-like signature is involved in the transcriptional regulation of genes that are expressed predominantly in root-tip regions (Ellis et al., 1993; Liu and Lam, 1994; Zhang and Singh, 1994; Droog et al., 1995a). This type of AuxRE is present in several promoters of viral and agrobacterial genes that are expressed in plants (Bouchez et al., 1989). Examples of real plant genes that contain such an *as-1/ocs*-like AuxRE in their promoter regions are the members of the auxin-inducible *Nt103/Nt107/Nt114* gene family of tobacco. These genes, encoding a particular class of GSTs (Droog et al., 1993, 1995b), were isolated from tobacco cell-suspension cultures (van der Zaal 1987, 1991). Independently several (nearly) identical genes, designated *par*, were isolated from auxin-treated tobacco mesophyll protoplasts (Takahashi et al., 1989; Takahashi and Nagata, 1992). The products of these *Nt103*-like genes were initially thought to be necessary for the process of auxin-induced cell division (van der Zaal et al., 1987; Takahashi et al., 1989). However, induction of these genes independently of a cell division response also appeared to be possible, in response to SA and an elicitor present in yeast extract (Boot et al., 1993; Boot, 1994). Induction of gene expression via *as-1/ocs*-like elements was also shown to occur in response to stimuli other than auxin treatment, such as SA, methyl jasmonate, and wounding (Kim et al., 1993, 1994; Qin et al., 1994; Zhang and Sing, 1994). The biological significance of induction by auxin and SA via *ocs* elements was recently challenged when it was found in a transient expression system that *ocs* elements were activated not only by biologically active auxins and SA but about equally well by biologically inactive analogs (Ulmasov et al., 1994).

Until now the signal transduction pathway leading to gene expression via *as-1/ocs*-like elements was not understood. Nuclear factors resembling or identical to ASF-1 (Lam et al., 1989) or OCSTF (Tokuhisa et al., 1990) have been shown to bind to these elements *in vitro*. Cloned bZIP

¹ This work was funded in part by the European Communities' BIOTECH Programme, as part of the Project of Technological Priority 1993–1996.

* Corresponding author; e-mail zaal@rulsfb.leidenuniv.nl; fax 31–71–5274999.

Abbreviations: AuxRE, auxin-responsive promoter elements; BA, benzoic acid; 3H-BA, 3-hydroxy-benzoic acid; bZIP, basic Leu zipper; CaMV, cauliflower mosaic virus; GST, glutathione *S*-transferase; LS, Linsmaier-Skoog; MU, methylumbelliferone; NAA, naphthylacetic acid; POA, phenoxyacetic acid; SA, salicylic acid (2-hydroxy-benzoic acid).

transcription factors such as TGA1a (Katagiri et al., 1989) and G13 (Fromm et al., 1991) and related proteins could be part of these nuclear factors in vivo. Because the bZIP class of transcription factors are active as homo- or heterodimers and multiple genes encoding such factors are present in the genomes of higher plants, the question of which dimer is actually binding to a promoter element in vivo is difficult to solve experimentally. Recently it was reported that the 35S promoter is active in the yeast *Saccharomyces cerevisiae* (Rüth et al., 1992) and can be further *trans*-activated by co-expression of cloned TGA1a (Rüth et al., 1994). A mutation in the *as-1* sequence that abolished TGA1a binding also inhibited the *trans*-activation. Such results using a yeast system could be regarded as evidence that the studied factor at least has the potential to be regulatory in the homologous system.

In this study, we wanted to investigate the *cis*-acting potential of the rather aberrant *as-1/ocs*-like sequences present in the promoters of tobacco auxin-inducible genes belonging to the *Nt103* and *Nt114* family (*as103* and *as114*, respectively) compared to the consensus sequence TGACGTAAGCGATGACGTCA (*ascon*). The general term *as* element acknowledges both the *as-1* element of the CaMV 35S promoter and the possible auxin sensitivity of the elements. As will be demonstrated, the elements appear to have no essential differences apart from their relative strength in yeast as well as in plant cells. The strength of the element is proportional to its affinity for TGA1a or related proteins.

MATERIALS AND METHODS

Plasmids

Oligonucleotides spanning the 20-bp *as* elements flanked by *Hind*III and *Bam*HI sticky ends (Pharmacia) were ligated into *Hind*III/*Bam*HI-digested pSN104 (Neuteboom, 1994). This construct in pUC8 (Viera and Messing, 1982) harbors the -55/+128 region (relative to translational start) of the *Agrobacterium tumefaciens* T-*cyt* gene (Neuteboom et al., 1993) translationally fused to the reporter gene *gusA* with a nopaline synthase terminator obtained from pBI101.3 (Jefferson, 1987).

The resulting plasmids contained the following sequences 16 bp upstream of the T-*cyt* TATA box:

as103: AGCTTATAGCTAAGTGCTTACGTATGGATC
as114: AGCTTTTACGCAAGCAATGACATCTGGATC
ascon: AGCTTTGACGTAAGCGATGACGTCAGGATC

The underlined sequences are present in the *Nt103-1* gene (and in the *Nt103-35* gene starting with T instead of A; van der Zaal et al., 1991) around -365 prior to ATG and in the *Nt114-4* gene (Droog et al., 1995a) around -96 prior to ATG. The consensus sequence is further based on published sequences (Ellis et al., 1993)

Plasmid pSN103 (Neuteboom, 1994) is derived from pSN104 by insertion of the CaMV 35S promoter (-523 to +5 relative to the transcriptional start site, Pietrzak et al., 1986), which was made available as a *Hind*III/*Bam*HI fragment and cloned into the corresponding sites. Plas-

mid pSN91 (Neuteboom, 1994) was derived from pSN104 by insertion of the -283/-80 part of the T-*cyt* promoter sequence.

For expression studies in yeast, the chimeric GUS constructs present in the plasmids described above were cloned as *Hind*III/*Eco*RI fragments in similarly digested YEplac112 (Gietz and Sugino, 1988). Because of the presence of an extra *Eco*RI site upstream of the 35S promoter in pSN103, this GUS construct was cloned as an *Eco*RI fragment. Since these initial constructs, especially those without a promoter, led to a very high GUS activity in yeast, a *Hind*III fragment of the central region of phage λ (nucleotides 25157-27479 on the λ map) was cloned into the *Hind*III sites upstream of all constructs (the 25157 position being most proximal to the GUS genes). The constructs eventually obtained were called YEas103, YEas114, YEascon, YE55, YE283, and YE35S.

The plant transcription factor TGA1a was made available from pKT7T1A (Katagiri et al., 1990) as an *Nde*I/*Xho*I fragment. The *Nde*I site just upstream of the ATG initiation codon was filled in using Klenow enzyme prior to *Xho*I digestion. The fragment was cloned between the yeast *ADH1* promoter and terminator sequences of *Not*I (filled in)/*Xho*I-digested YEP181A1MOD. This latter vector was obtained from YEP181A1NE (Riesmeier et al., 1992) by modification of the polylinker sequence mainly for other purposes. Briefly, plasmid YEP181A1NE was digested with *Pst*I and *Sma*I, treated with T4 DNA polymerase, ligated, and used to transform *Escherichia coli* strain XL1-Blue (Stratagene). After the plasmid was isolated, the DNA was digested with *Bam*HI and filled in with Klenow enzyme, and *Xho*I-*Eco*RI adaptors (Stratagene) were ligated to the blunt ends. Following T4 polynucleotide kinase treatment and gel purification, the vector DNA was religated and transformed again. The resulting YEP181A1MOD thus contained a new *Xho*I site next to the terminator. The plasmid containing the TGA1a-coding sequence was designated YEP181A1MOD.TGA1.

Yeast Strains

Yeast strain YPH499a (*ura3*, *lys2*, *ade2*, *trp1*, *his3*, *leu2*; Sikorski and Hieter, 1989) was first transformed with plasmids YEP181A1MOD and YEP181A1MOD.TGA1 by electroporation (Becker and Guarente, 1991). Transformants were selected on minimal-yeast medium (Zonneveld, 1986) lacking Leu. Because the vector-transformed yeast strain served as a control, it was designated YE-CON; the TGA1a-containing strain was designated YE-TGA. Both strains were transformed next with the plasmids YEas103, YEas114, YEascon, YE55, YE283, and YE35S and selected on plates lacking Leu and Trp. These strains were used for determination of GUS activity.

Determination of GUS Activity

Yeast cells were grown in minimal-yeast medium (lacking the appropriate amino acids for plasmid selection) overnight at 28°C. The optical density at 600 nm was usually approximately 0.7, and cells were diluted or concen-

trated by centrifugation to reach the same optical density (i.e. same amount of cells). After centrifugation, the medium was removed by aspiration and the cell pellets were frozen in liquid nitrogen. After the sample was thawed, GUS extraction buffer (Jefferson, 1987) was added and cells were vortexed for 1 min. Appropriate amounts of the permeabilized yeast cells were added to buffer containing 4-methyl-umbelliferyl β -D-glucuronide to determine GUS activity (Jefferson, 1987).

Plant Cell Transformation

Chimeric GUS genes present in the plasmids YEas103, YEas114, Yeascon, YE55, and YE283 were gel purified after digestion with *EcoRI* and cloned into the wide host range vector pMOG22 (Goddijn et al., 1993). Clones with the remaining 950 bp of the λ fragment (*EcoRI* site at 26104 within fragment 25157-7479 of phage λ) adjacent to the right border were selected. After the plasmids were mobilized into *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) using a triparental mating procedure (Ditta et al., 1980), tobacco BY2 cells were transformed by the co-cultivation method (An, 1985). Of the 4 mL of BY2 culture used originally, half was transferred after 3 d to solid LS medium (Linsmaier and Skoog, 1965) containing cefotaxime and vancomycin (100 μ g/mL each) to kill remaining bacteria and 25 μ g/mL hygromycin to select for transformed cells. The other half was transferred directly to liquid LS medium containing the same compounds. For both media, the concentration of 2,4-D was 0.05 mg/L. Cells were grown at 28°C in the dark on a gyratory shaker in 20 mL of medium in 100-mL flasks with aluminum caps. After 2 to 3 weeks and several changes of the liquid medium, readily growing cell suspensions were obtained, which were transferred at weekly intervals (using 1.5–2 mL of inoculum). On solid medium, hundreds of calli started to develop, indicating that transgenic cell suspensions were a mixture of many independent transformants. After 3 months, cells were free of bacterial contamination, as determined by plating some of the culture on bacterial agar plates and incubating for 1 week at 28°C, and antibiotics were omitted from the cell suspensions.

Induction Experiments with Transformed Tobacco Cells

Early stationary-phase cells were diluted 10- to 12-fold in LS medium without any hormones, and 0.5-mL portions were rapidly transferred to wells of a 24-well plate (Becton Dickinson) containing a small amount of concentrated solution of the compound to be tested for induction of gene expression (usually between 5 and 25 μ L of 100 \times to 20 \times concentrated solutions). The weakly acidic solid compounds used were dissolved in 0.1 M KOH in such a way that the molar amount of KOH equalled the molar amount of the compound to be dissolved. When a clear solution was obtained, it was adjusted to pH 6 by adding diluted KOH or HCl. Stock solutions of 5 to 10 mM were stored at -20°C . Cells and compounds were rapidly mixed. The well plates were kept slightly open by taping toothpicks to the underside of the lids.

After the plates were shaken at 28°C in the dark for the times indicated for the experiments, they were put on ice, and after addition of 1 mL of ice-cold demineralized water, the cells were rapidly transferred to 1.5-mL reaction tubes. Liquid was removed by aspiration through a narrow glass pipet, and cells were frozen in liquid nitrogen. For GUS measurements, 0.1 mL of extraction buffer (Jefferson, 1987) was added and cells were ground by 10 strokes with a potter (set at 1500 rpm) just fitting the tube. Portions of the supernatant were used to determine GUS activity.

Fluorescence was measured by using an automated plate reader (Perkin-Elmer LS50) and was standardized against dilutions of Na⁺ MU. Protein concentrations in the extracts were determined (Bradford, 1976) using BSA as a standard. All experiments were performed in duplicate and repeated several times. Routinely, 90% of the duplicate measurements differed by less than 10%.

Gel-Shift Analysis

Protein extracts were prepared from yeast cells as described by R \ddot{u} th et al. (1994) except that 2 volumes of saturated ammonium sulfate solution were used to precipitate the proteins. For the isolation of nuclei from cell cultures, early stationary-phase BY2 cells (from one 50-mL culture that was treated with 2 μ M 2,4-D for 15 min) were first protoplastized in 0.4 M mannitol containing 1% cellulase RS and 0.1% pectolyase Y23 for 2 h in the dark at 28°C. After several washes with 0.4 M mannitol, the protoplasts were resuspended in 5 mL of 0.4 M mannitol and subsequently lysed by rapid dilution with an equal volume of 20 mM MgCl₂, 50 mM Hepes, pH 7.8, containing 1.6 mM PMSF. After the sample was centrifuged at 500g for 5 min at 4°C, the pellet was resuspended in 5 mL of ice-cold 0.2 M mannitol, 10 mM MgCl₂, 25 mM Hepes, pH 7.8, 0.8 mM PMSF. After addition of Triton X-100 to 0.5% (v/v) and gentle swirling, the suspension was layered on top of 40 mL of buffer A (25 mM Hepes, pH 7.8, 20 mM KCl, 20 mM MgCl₂, 0.6 M Suc, 40% [v/v] glycerol, 10 mM 2-mercaptoethanol, 0.8 mM PMSF, 0.5% [v/v] Triton X-100) in a 50-mL Falcon tube. After the sample was centrifuged at 4000g for 20 min at 4°C the nuclear pellet was taken up in a small volume of buffer A lacking Triton X-100 and concentrated by centrifugation in an Eppendorf tube. Nuclei were snap frozen in liquid nitrogen and stored at -80°C . Protein extracts were made from these preparations using the buffers and procedures that were used for the preparation of yeast protein extracts (R \ddot{u} th et al., 1994). Vortexing with glass beads was omitted. Nuclear extracts from tobacco leaves were prepared according to published procedures (Green et al., 1989).

Labeled *as103* and *ascon* elements were obtained by first annealing the complementary primers and then filling in the protruding 5' ends by use of the Klenow fragment of DNA polymerase I in the presence of [α -³²P]dCTP. Gel-shift conditions were as described by R \ddot{u} th et al. (1994); 20- μ L reactions contained 0.1 ng of labeled fragments and 2 to 5 μ g of protein, always added as the last component. For competition, a 100-fold amount of cold fragment was added. Polydeoxyinosinic-deoxycytidylic acid was present

at 2 μg per reaction. After 20 min of incubation at room temperature, reactions were loaded and electrophoresed on a 6% polyacrylamide gel in 0.5 \times Tris-borate-EDTA buffer.

Synthesis of 2,4-D Analogs

Analogs of 2,4-D were prepared according to standard methods starting from the appropriate dichlorinated phenols (Sigma). Briefly, 1.63 g of the dichlorinated phenol were heated with 0.945 g of chloroacetic acid in 6 mL of water at 90°C for 15 min. After addition of 4 mL of 5 N NaOH the mixture was heated for 3 h at 98 to 100°C. After addition of 1.9 mL of concentrated HCl, the mixture was allowed to cool and the formed crystals were collected and rinsed with distilled water. The compounds were recrystallized three times from a 250-mL volume. The crystals were collected and dried under vacuum. Yields ranged from 4 to 35% of the theoretical maximum. An extra sample of 3,5-dichlorophenoxyacetic acid was a gift from Prof. H. Veldstra.

RESULTS

Expression in Yeast

We wanted to investigate the potential differences between *as* elements (*as-1/ocs*-like elements) present in the auxin-inducible promoters of the *Nt103*-like *gst* genes of tobacco (*as103*, *as114*) and a consensus sequence of all these elements (*ascon*). A rapid procedure for this goal could be the testing of different elements upstream of a heterologous TATA box in yeast cells. A test for the functional interaction of tobacco bZIP transcription factor TGA1a with the different elements could provide an extra criterion for the classification of *as* elements (Rüth et al., 1994).

All constructs that were used for the experiments are shown in Figure 1. The minimal promoter present in YE55, having just a TATA box, was derived from the *T-cyt* gene of *A. tumefaciens*. This minimal promoter upstream from the chimeric GUS gene never gave rise to detectable GUS activity in transgenic plant material, whereas a longer promoter, such as that present in YE283, led to high levels of GUS activity (Neuteboom et al., 1993; Neuteboom, 1994). We found it necessary to clone a DNA fragment from phage λ as a buffer upstream of the different promoters that we tested because quite often sequences around

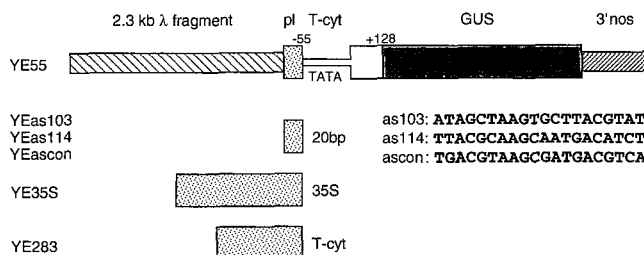


Figure 1. Schematic drawing of constructs used for yeast transformation. Relevant 20-bp sequences present in YEAs103, YEAs114, and YEAscon are shown.

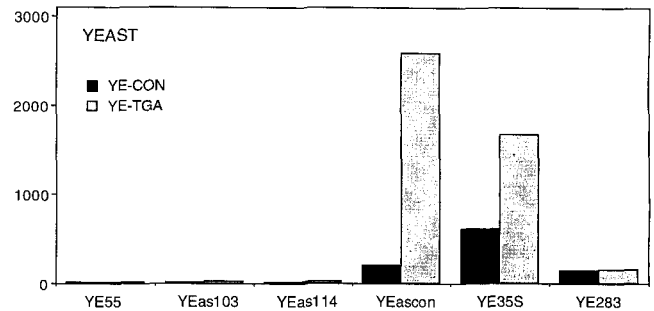


Figure 2. GUS activity in yeast cells. GUS activity (in arbitrary units) of cells containing the different promoter-*gusA* fusions of the plasmids indicated was determined after overnight growth in minimal medium. Dark bars represent GUS activity in yeast cells also producing plant TGA1a (YE-TGA); lighter bars represent GUS activity in yeast cells without extra TGA1a (YE-CON).

polylinkers of yeast plasmids appeared to be potent activators of gene expression in yeast (B.J. van der Zaal, F.J. Pieterse, unpublished results).

The context in which the different 20-bp *as* sequences were embedded eventually turned out to be sufficiently inert to allow experimental determination of differences between the elements tested. Construct YE55, without an *as* element, led only to background levels of GUS activity (Fig. 2). Activity of the *T-cyt* promoter in YE283 was considerable but was not enhanced by TGA1a. Since the *T-cyt* promoter lacks an *as* element, this lack of *trans*-activation was expected. The results with the 35S promoter positive control YE35S agreed very well with published data (Rüth et al., 1992, 1994). The activity of this promoter is already quite high in yeast, and since the *as-1* sequence is present within the promoter, the expression is enhanced by the presence of TGA1a protein (about 3-fold in our experiments). Of the different *as* elements that we tested, the consensus element *ascon* was most active. When no plant TGA1a was present in the yeast strain, the activity due to this element was already about 35% of the activity reached by the 35S promoter. Expression of TGA1a greatly enhanced (about 10-fold) the activity of the *ascon*-containing promoter, to levels beyond that of the 35S promoter. In contrast, the *as103* and *as114* elements displayed activities that led to GUS levels of approximately background values, similar to the construct lacking an element (YE55) or untransformed yeast. Only a slight enhancement of GUS activity could be observed in the presence of TGA1a. In relative terms, however, this induction could still be quite large, but in this situation the relative fold induction could not be calculated.

It thus has become clear that a single *as* element, *ascon*, can be a good promoter core for endogenous yeast *trans*-acting factors, whereas the other elements, which deviate from the consensus sequence, are active at a much lower level. For all *as* elements tested here, the plant protein TGA1a was able to enhance the level of gene expression brought about by the elements. Whether the difference in the level of gene expression reflects the need for different *trans*-acting factors or the strength of the interaction be-

tween the same factors with the different sequences cannot be concluded from the experiments in yeast cells.

Expression in Plant Cells

The chimeric GUS genes that were analyzed in yeast cells were also expressed in cultured BY2 tobacco cells. This would enable us to determine both relative strength of the *as* elements and the induction of gene expression via these elements by different auxin-like compounds and other treatments. For technical reasons the 35S promoter fusion was omitted. We preferred the use of transgenic cell suspensions over transgenic plants because the *Nt103* genes were originally isolated from a cell-culture system (van der Zaal et al., 1987). Induction experiments *in vivo* have several drawbacks, such as the need to compare many independent transformants to verify observed changes in gene expression and the complexity due to the use of different types of cells (Droog et al., 1995a). The stably transformed cell suspensions were derived from hundreds of independent transformants. Consequently, the data obtained using this material were expected to represent average values for reporter gene expression that are not, or are only mildly, dependent on positional effects of the GUS genes in the genome of individual transformants. During the experiments with the cell cultures spread over more than 6 months, no trend toward other expression strengths was observed. This indicated that the mixed population of transformants retained sufficient complexity. A cell-suspension culture that was made using the same method and contained the full-length *Nt103-35* promoter fused to *gusA* (van der Zaal et al., 1991) has been maintained for several years in our laboratory without noticeable changes. This cell line, *Nt103-35-GUS*, was used in this study to investigate which induction characteristics of the full-length promoter were conserved on the different *as* elements.

After comparison of the GUS activity levels of the different cell lines (Fig. 3), it became clear that of all of the *as* elements tested, the *as103* element led to the lowest levels

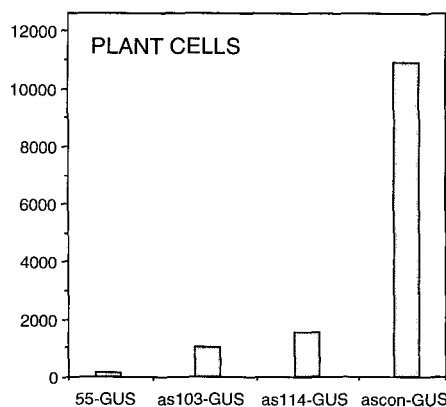


Figure 3. GUS activity in stably transformed plant cells. GUS activity (pmol MU mg⁻¹ protein min⁻¹) was measured in early stationary-phase tobacco BY2 cells transgenic for chimeric genes with the *as* elements indicated or the construct containing just a polylinker sequence in the promoter region (55-GUS). The data are mean values from a representative experiment (see Fig. 4) carried out in duplicate.

of GUS activity. The *as114* element appeared to be a little stronger, but both of these naturally occurring elements were about 10-fold weaker than the ideal element, *ascon*. The relative order of strength of the elements was thus remarkably similar in yeast and plant cells (compare data in Figs. 2 and 3). When challenged with a variety of different dichlorinated POAs and other compounds, as indicated in Figure 4, the behavior of the *as* element constructs was found to be qualitatively very similar. As shown in Figure 4B, the *as103* and *as114* elements can hardly be distinguished, except for a slightly higher general activity of the *as114* element after all treatments. For the *ascon* element (Fig. 4C), the relative induction of gene expression by several compounds was lower. In absolute terms, however, the increase seen after various treatments was larger for the stronger element. For all experiments with *as* elements there was no essential difference among induction times ranging from 4 to 8 h. The T-*cyt* promoter was not affected by any of the tested compounds in a reproducible manner (data not shown).

Based on the results mentioned above, we believe that all tested elements are equal with respect to inducibility characteristics. It is the amount of basal, uninduced activity that reveals the differences between the elements. Hence, the relative fold induction of gene expression by certain stimuli is simply dependent on the basal activities conferred by different elements, which could easily vary greatly between different test systems.

Inducer Specificity

Further comparisons between cell line *Nt103-35-GUS* containing the full-length promoter (Fig. 4A), and the cell lines containing *as*-element-driven minimal promoters (Fig. 4, B and C) revealed that, generally, compounds that induced the complete promoter were also able to induce gene expression through the *as* elements. The larger relative changes in GUS activity in *Nt103-35-GUS* cells compared to those in cells with the minimal *as* promoters were caused primarily by the less-leaky character of the full-length promoter in the uninduced situation.

Induction of gene expression was not correlated with the activity of the compound as an auxin. For 1-NAA (active auxin) and 2-NAA (inactive auxin), neither the full-length promoter nor one of the *as* elements was able to positively identify the active auxin 1-NAA. Some specificity was observed for the full-length promoter with the series of dichlorinated POAs, because the most active auxin, 2,4-D, led to the highest level of GUS activity. In the case of cell lines containing only *as*-element-driven minimal promoters, however, inactive 2,4-D analogs induced equally well. The elements appeared to have lost all specificity for induction by biologically active and inactive compounds in agreement with recent data (Ulmasov et al., 1994). The elicitor present in yeast extract led to only a very modest induction of GUS activity via the *as* elements, whereas it acted as an extremely potent inducer of the full-length *Nt103-35* promoter (Fig. 4A; Boot, 1994). Surprisingly, SA induction also appeared not to be mediated by the *as* elements, although similar elements in leaves of transgenic plants have been

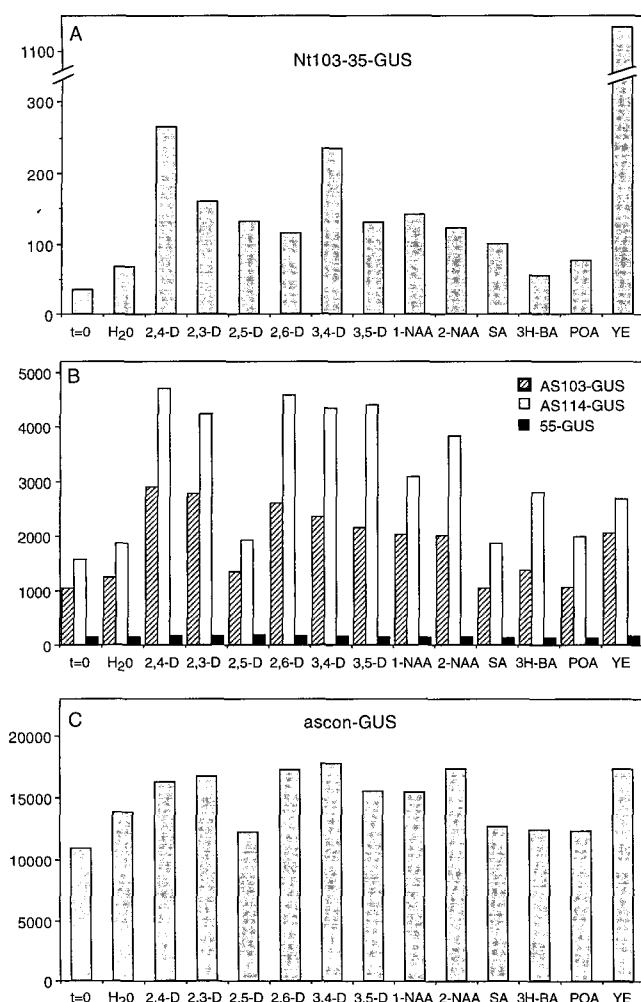


Figure 4. Effect of different compounds on gene expression. GUS activity ($\mu\text{mol MU mg}^{-1} \text{protein min}^{-1}$) was measured in BY2 cells after 8 h of treatment with the compounds indicated at $5 \mu\text{M}$ final concentration, except for yeast extract (YE), which was present at 0.1% (w/v). The data are mean values from a representative experiment carried out in duplicate. The transgenic cell lines used are indicated in the panels. GUS activity at the start of the experiment is given as $t = 0$. *n,n*-D, *n,n*-dichlorophenoxyacetic acid.

reported to be SA inducible (Kim et al., 1993; Qin et al., 1994).

For several compounds and weak acid controls, a partial dose-response curve was established using the *Nt103-35-GUS* cell line and the cell line containing the minimal promoter driven by the *as103* element (Fig. 5). The data calculated for each independent replication using the *Nt103-35-GUS* cells were in absolute terms rather variable, because of the comparatively low levels of GUS activity in these cells. This resulted in rather large sds (Fig. 5A), but the induction characteristics for the compounds were very similar in the different experiments. For reasons of convenience and to get more comparable figures for the two cell lines, the data were plotted as percentages of induction relative to the uncorrected activity (background plus GUS activity) of the sample taken at the start of the experiment.

It should be realized that a 50% relative increase for the *Nt103-35-GUS* cells easily represents a 6- to 8-fold induction of GUS activity in absolute units, whereas for the *as103* cells the difference between relative and absolute scales is marginal because of the much higher basal level of GUS activity (Fig. 4).

As can be seen in Figure 5 the observations made for the single-concentration experiments were further corroborated. For the two NAA analogs, both the full-length promoter and the *as103* element reacted similarly, whereas for the inactive auxin 3,5-dichlorophenoxyacetic acid, the induction of the full-length promoter was slightly less effective than for 2,4-D. For SA and its inactive analog 3H-BA, it became evident that the full-length promoter was specifically induced by SA, whereas for the *as103*-element-containing minimal promoter, 3H-BA was the better inducer. Generally, weak acids were active as inducers of the full-length promoter only at concentrations well above $10 \mu\text{M}$, whereas auxin analogs and SA were already leading to rather high levels of GUS activity at 10-fold lower concentrations.

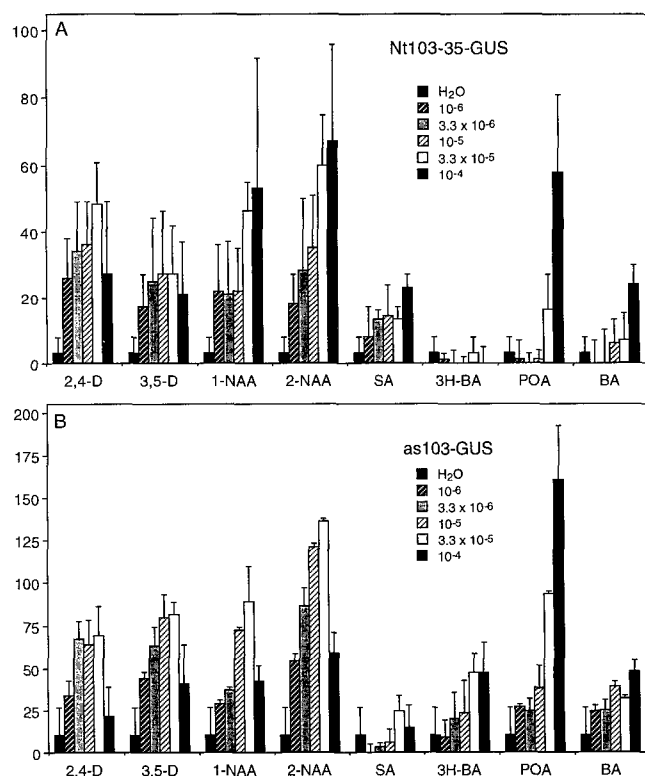


Figure 5. Dose-response plots for different inducers. GUS activity was measured after 6 h of treatment of BY2 cells containing the full-length promoter construct (*Nt103-35-GUS*, A) or the *as103* element containing minimal promoter (B) with the compounds shown at the concentrations indicated. Values are given as a percentage relative increase of GUS activity over the apparent GUS activity (including background) of the sample taken at the start of the experiment ($t = 0$). For further explanation, see the text. Error bars represent the sd calculated from three experiments (A) or the deviation from the mean of two experiments (B), all performed in duplicate. 3,5-D, 3,5-dichlorophenoxyacetic acid.

For the *as103* cell line, the difference between auxin-like compounds and weak acid controls was less pronounced but was clearly there. SA did not lead to significant induction of gene expression via the *as103* element up to the highest concentration tested, which was 0.1 mM. At this concentration, the weak acids BA and POA, as well as 3H-BA, as mentioned above, were more active inducers of the *as103* line. Several weak acid controls at different concentrations should thus be used to analyze the possibility that there is specifically induced gene expression under control of *as* elements. Moreover, in cell-suspension cultures, concentrations of membrane-permeable weak acids exceeding 10 μ M should be avoided.

In conclusion, in transgenic cell-suspension cultures the results obtained with *as* elements indicate that elements that differ in sequence still behave very similarly. As in yeast, the strength of the element is the most characteristic feature of an element. The relative strengths of *as* elements appear to be similar whether the elements are analyzed in yeast or in plant cells.

Gel-Shift Analysis

When it became clear from the experiments described above that their relative strength was the only clear difference between various *as* elements, we investigated whether this difference was reflected in the affinity of transcription factors for these elements. By means of gel-shift analysis, the interaction between proteins present in (nuclear) extracts from plant cells or yeast was analyzed. As demonstrated above, the cloned transcription factor TGA1a, when expressed in yeast cells, was able to positively affect the levels of gene expression of all constructs having an *as* element. The elements used for the experiments described here were the weakest element, *as103*, and the strongest element, *ascon*. There was indeed a very good correlation between the activity of the element and the binding of proteins to the element (Fig. 6A). Extracts from yeast cells that do not produce plant TGA1a contained virtually no proteins that gave a detectable shift with the *as103* element. Yeast containing TGA1a proved to contain protein that resulted in a significant shift of this element. The *ascon* element was already shifted by yeast extract without TGA1a, and this shift was further intensified when the TGA1a protein was present. Competition with unlabeled *as103* element only very weakly diminished the interaction, whereas unlabeled *ascon* element virtually abolished the shift obtained. In yeast, the expression levels reached by the constructs with *as* elements in the different strains (Fig. 2) thus correlated very well with the amount of protein binding to the elements in vitro. The presence of plant TGA1a, which, when expressed in yeast, greatly enhanced gene expression, also led to enhanced binding of protein to *as* elements. The most straightforward explanation for these observations is that TGA1a, when present in yeast, becomes at least one-half of the dimer of bZIP proteins that acts as a *trans*-acting factor for the *as* elements.

When the *as* element-binding activity from extracts of TGA1a-containing yeast cells was compared with the binding activity present in nuclear extracts from plant cells

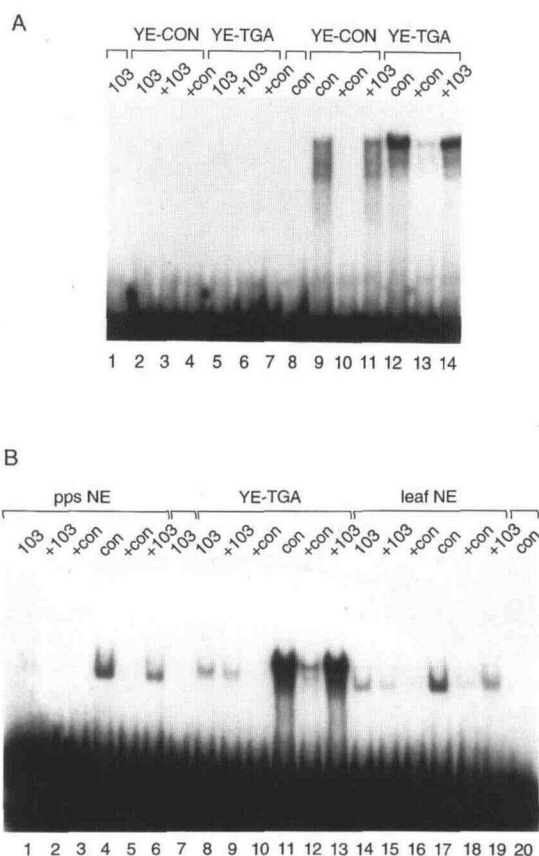


Figure 6. Gel-shift analysis. A, Extracts from yeast cells producing plant transcription factor TGA1a (YE-TGA) and extracts from control cells (YE-CON) were tested for their ability to bind to labeled *as103* (103) or *ascon* (con) elements as indicated. Lanes 1 and 8 contained DNA without addition of protein (free probe). In lanes marked with +103 or +con, a 100-fold excess of unlabeled *as103* or *ascon* was added. B, Comparison between nuclear extracts from tobacco protoplasts/plant cells (pps NE), tobacco leaves (leaf NE), and extract from TGA1a-producing yeast cells (YE-TGA) for their ability to bind labeled *as103* (103) or *ascon* (con) elements. Lanes 7 and 20 contained the free probes. As in A, in the lanes marked with +103 or +con, a 100-fold excess of unlabeled *as103* or *ascon* was added. Exposure time for the gel in B was longer than for the gel in A.

(derived from cultured cells and leaves), a similar picture emerged (Fig. 6B). The *as103* element was only weakly bound by protein and was a poor competitor for binding. The *ascon* element bound protein with much higher affinity and was also a very good competitor. Both in TGA1a-containing yeast cells and in plant cells, a DNA-binding activity with strikingly similar specificity was present. Extending the results obtained with protein extracts from yeast, TGA1a thus appeared to be (part of) the complex binding to *as* promoter elements in plant cells too. It must be noted, however, that based on the results described above, no firm conclusions can be drawn concerning the exact identity of the protein(s) constituting the transcription factor from plant cells that binds to the *as* elements. The slightly lower mobility of the retarded complexes when extracts from yeast are used instead of extracts from plant nuclei (Fig. 6B) seems to favor the conclusion that the

proteins present in the complexes are different. However, unknown differences in protein modification between yeast and plant cells might also be the cause of different mobility in a gel-shift assay. TGA1a (or an effectively very much related bZIP protein) is at least a very good candidate to be part of the factor that mediates gene expression via *as* elements.

The binding strength between the transcription factor and the *as* element, as judged by the gel-shift analysis, seems to determine the strength of the element for activation of gene expression. Other possibilities for the regulation of the strength of an *as* element can be created by the dual TGACG motifs within an *as* element. When both motifs are strongly bound by the transcription factor, the *cis*-activating potential of such an element will increase. The strong *ascon* element can efficiently bind a second transcription factor, thus a second dimer of bZIP proteins, as is evident from the presence of a slower-migrating complex in the gel-shift assay (Fig. 6; this is especially clear in lane 12 of Fig. 6B). The *as103* element has rather poor TGACG motifs, especially the one at the 5' end; therefore, it does not easily bind more than one transcription factor and forms predominantly the faster-migrating complex.

DISCUSSION

We have shown that 20-bp TGACG[N7]TGACG-like elements that are present in auxin-inducible promoters of tobacco *gst* genes functionally resemble the more widely studied *as-1*, or *ocs*, element of the CaMV 35S, or octopine synthase promoter of *A. tumefaciens*. Such elements have been reported to be important for auxin-inducible gene expression (Liu and Lam, 1994) and for inducibility of gene expression by other plant signal molecules, such as SA and methyl jasmonate (Kim et al., 1993, 1994; Qin et al., 1994; Zhang and Singh, 1994). For historical reasons and for the sake of clarity, we refer to these elements as *as* elements, thus acknowledging both the *as-1* element and the auxin sensitivity of these elements.

As a first conclusion from our study, it has become clear that both in yeast cells and in transgenic cell-suspension cultures of tobacco the *as* elements are important *cis*-acting elements for gene expression. As a second conclusion, we can say that the difference in relative strength of the *as* elements is the only clear difference observed among them. A strong element for yeast cells is also a strong element for plant cells. The third conclusion is that the strength of a particular *as* element is correlated with the strength of the interaction of the elements with nuclear proteins. Tobacco bZIP protein TGA1a or a closely related transcription factor is a very likely candidate to bind to these elements *in vivo* and thus to enhance gene expression.

According to our data, *as* elements should be regarded as basically similar except for their intrinsic strength of *cis*-acting activity. Yeast cells proved to be a very useful model system in which to study the relative strength of *as* elements, since the response in yeast was very similar to that in stably transformed plant cells. The functional interaction of TGA1a expressed in yeast with *as* elements confirmed results obtained with the 35S promoter (Rüth et al., 1994).

Thus in principle screening for *trans*-acting factors (other than TGA1a) can be performed in yeast cells using a genetic screening method (Wang and Reed, 1993). So far we have not found *trans*-acting factors that bind the weak *as103* element with higher affinity than TGA1a.

Perhaps proteins recognizing TGACG[N₇]TGACG-type motifs, whether they are yeast or plant proteins, do so on a more general structural basis also determined by the 7-bp intervening sequence (Kim et al., 1994). A nomenclature based on the ACGT core within the binding sites recognized by plant bZIP proteins (Izawa et al., 1993; Foster et al., 1994) would describe the *as103* element as two A boxes spaced 7 bp apart. The first A box of this element has an AGCT core instead of ACGT. Similarly, the *as114* element has an A and a C box, both lacking the ACGT core. The *ascon* element comprises a C/A box and a C box, both with a consensus ACGT core, and the 35S *as-1* element consists of two C/A boxes of which the 3' one has an ACGC core. The *ocs* (Ellis et al., 1987) and *nos* elements (Lam et al., 1990) are composed of T/A and A boxes and C/A and A/C hybrid boxes, respectively. Generally, all elements that have been found to be auxin inducible in this and other studies are composed of two A or C boxes or hybrid boxes with A and C half sites.

It has been predicted that genes possessing high-affinity C, A, or A/C hybrid boxes are regulated by so-called group-3 factors (Foster et al., 1994). The bZIP protein TGA1a is so far the only protein that has been placed in this group. If it is indeed TGA1a or a highly similar protein that is binding to the *as* elements used in our study, this would imply that TGA1a does not strictly require an ACGT core, because the *as114* element lacks such a core. Indeed, it has recently been shown that this is the case, although elements without an ACGT core are bound with decreased affinity (De Pater et al., 1994). An increase in DNA-binding strength by the posttranscriptional modification of the *trans*-acting factor could very well provide a mechanism for transcriptional activation. Having a variety of related *cis*-acting elements and *trans*-acting factors would thus enable each particular plant cell to achieve induction of gene expression by certain compounds. The apparent redundancy of TGA1a-like proteins in plants (Miao et al., 1994) fits well into such a model.

Our results obtained with transgenic cell-suspension cultures corroborate that *as* elements are *cis*-acting sequences important for transcriptional activation of gene expression upon treatment with auxin-like compounds. However, in contrast to results obtained by others using mostly leaves of transgenic plants (Kim et al., 1993, 1994; Qin et al., 1994; Zhang and Singh, 1994), we found that the *as* elements were not important for SA-induced gene expression. The biological relevance of both the auxin- and SA-inducible gene expression via *as* elements has rightfully been questioned recently because no comparison was made with structural analogs lacking appropriate biological activity. In a transient heterologous expression system, it was found that an *as* element from a soybean promoter was activated by both active and inactive auxin and SA analogs (Ulmasov et al., 1994). Considering SA-induced gene expression, we

found that the full-length *Nt103-35* promoter was induced only by the active compound, whereas SA itself clearly did not induce via the *as103* element (Fig. 5). Therefore, the results from our study indicate that SA inducibility present in the full-length promoter is not as such present in the *as* elements and neither is inducibility by elicitors present in yeast extract.

Considering the auxin responsiveness of the *as* elements, we could confirm the lack of true auxin specificity observed for the *as* element of an auxin-inducible promoter from soybean (Ulmasov et al., 1994). Induction of gene expression in plants by auxin-like compounds via *as* elements might be an example of the more widely found induction of GSTs by electrophilic compounds. Electrophilic response elements are present within the promoters of certain mammalian *gst* genes, and they do have a structural resemblance to plant *as* elements, as noted before (Ulmasov et al., 1994; Zhang and Singh, 1994). However, it is still possible that in plants an endogenous compound resembling or identical with auxins is responsible for gene expression via *as* elements predominantly in root tips. Lack of specificity found for exogenously applied inducers of gene expression can be caused by the fact that the compounds used are poor mimics of the endogenous compound and/or by differences in uptake and stability. For this reason, it is not yet clear from what kind of lack of specificity the induction of gene expression via *as* elements could possibly suffer. Elucidation of the signal transduction pathway(s) leading to enhanced gene expression via *as* elements could prove to be very valuable for understanding more of the elementary processes taking place in plants.

ACKNOWLEDGMENTS

We thank Jan Schouten for his excellent advice during the yeast experiments, Pieter Ouwerkerk for nuclear extract from tobacco leaves, Dr. F. Katagiri for the gift of plasmid pKT7T1A, and Dr. W.B. Frommer for plasmid YEP181A1NE. We thank Peter Hock for preparation of figures and Adri 't Hooft for photography.

Received August 1, 1995; accepted October 5, 1995.

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