The Plant Cell, Vol. 6, 1087–1098, August 1994

A Dominant Negative Mutant of PG13 Suppresses Transcription from a Cauliflower Mosaic Virus 35S Truncated Promoter in Transgenic Tobacco Plants

Mechthild Rieping, Markus Fritz, Salome Prat,[1] and Christiane Gatz[2]

Universität Bielefeld, Lehrstuhl für Genetik, Fakultät für Biologie, 33501 Bielefeld, Germany

TGA1a and PG13 constitute a family of tobacco basic leucine zipper (bZIP) proteins that bind to activating sequence-1 (*as-1*), which is one of the multiple regulatory *cis* elements of the cauliflower mosaic virus (CaMV) 35S promoter. After truncation of the CaMV 35S promoter down to position −90 (CaMV 35S [−90] promoter), transcription stringently depends on the presence of *as-1*, which is recognized by nuclear DNA binding proteins called ASF-1. The role of the TGA1a/PG13 bZIP family in the formation of ASF-1 and in transcriptional activation of the CaMV 35S (−90) promoter has not yet been demonstrated in vivo. We constructed transgenic tobacco plants expressing a mutant of potato PG13, which lacks its wild-type DNA binding domain. This mutant acts as a *trans*-dominant inhibitor of ASF-1 formation and of expression from the CaMV 35S (−90) promoter, showing that PG13 can specifically interact with proteins necessary for these processes. Although we did not observe any other obvious phenotypic changes, these transgenic plants are a potentially valuable tool in identifying whether TGA1a and PG13 are involved in controlling promoters encoded in the plant genome.

INTRODUCTION

The cauliflower mosaic virus (CaMV) 35S promoter confers almost constitutive expression upon heterologous genes in most plants. Dissection of the promoter region into subdomains that are able to confer tissue-specific gene expression has demonstrated that the promoter has a modular organization (Benfey and Chua, 1990). Deletion of sequences upstream of position −90 relative to the transcription start site limits its expression to root tips, callus, protoplasts, and auxin-induced leaves of transgenic tobacco plants (Benfey et al., 1989; Fromm et al., 1989; Liu and Lam, 1994). This expression pattern is a result of activating sequence-1 (*as-1*), a 17-bp *cis* element located 34 bp upstream of the TATA-box. Deletion of this sequence within the full-length CaMV 35S promoter does not affect transcription in leaves (Benfey et al., 1989). However, mutations in *as-1* abolish the modest increase in the expression level of the wild-type CaMV 35S promoter that is observed when leaves were treated with auxin (Liu and Lam, 1994). *as-1* contains two TGACG motifs; they interact with ASF-1, a complex of proteins in nuclear extracts from leaves (Lam et al., 1989; Prat et al., 1989). Using the TGACG motif as a probe, Katagiri et al. (1989) isolated two cDNA clones (*tga1a* and *tga1b*) from tobacco, whose gene products (TGA1a and TGA1b) bind specifically to *as-1*. The tobacco *TGA1a* cDNA served as a probe to isolate a different but related gene, named *g13*, from a tobacco genomic library (Fromm et al., 1991).

The primary structure of TGA1a, TGA1b, and PG13 (the gene product of *g13*) predicts that these proteins contain a basic leucine zipper (bZIP) domain, which is a well-defined DNA binding motif found in several eukaryotic transcriptional activators (Landschulz et al., 1988; McKnight, 1991). This domain dimerizes by forming a coiled-coil structure in the leucine zipper (ZIP) region. Dimerization is essential for DNA binding. Katagiri et al. (1992) have performed deletion analyses of TGA1a to prove the function of the predicted domains (Figure 1A). The deduced ZIP region of the protein was necessary and sufficient for dimerization. DNA binding depended on the presence of the whole bZIP region. Dimer formation was stabilized by the so-called dimer stabilization region between amino acids 178 and 373. Generally, a bZIP protein can form dimers with a distinct subset of bZIP proteins. Heterodimer formation constitutes an important tool for transcriptional control mechanisms (Lamb and McKnight, 1991).

The roles of TGA1a, TGA1b, and PG13 in the regulation of the CaMV 35S (−90) promoter are still not understood. The primary structure of TGA1a and PG13 reveals an acidic region at the N terminus and a glutamine-rich region located in the C-terminal portion of the protein (Figure 1A), suggesting that these proteins might be transcriptional activators (Katagiri et al., 1989). TGA1a has been shown to function as an activator in vitro, when two *as-1* binding sites were located upstream of the TATA-box (Katagiri et al., 1990; Yamazaki et al., 1990).

[1] Current address: Centro de Investigacion y Desarrollo, Consejo Superior de Investigaciones Cientificas, Jorge Girona 18-26, 08034 Barcelona, Spain.

[2] To whom correspondence should be addressed.

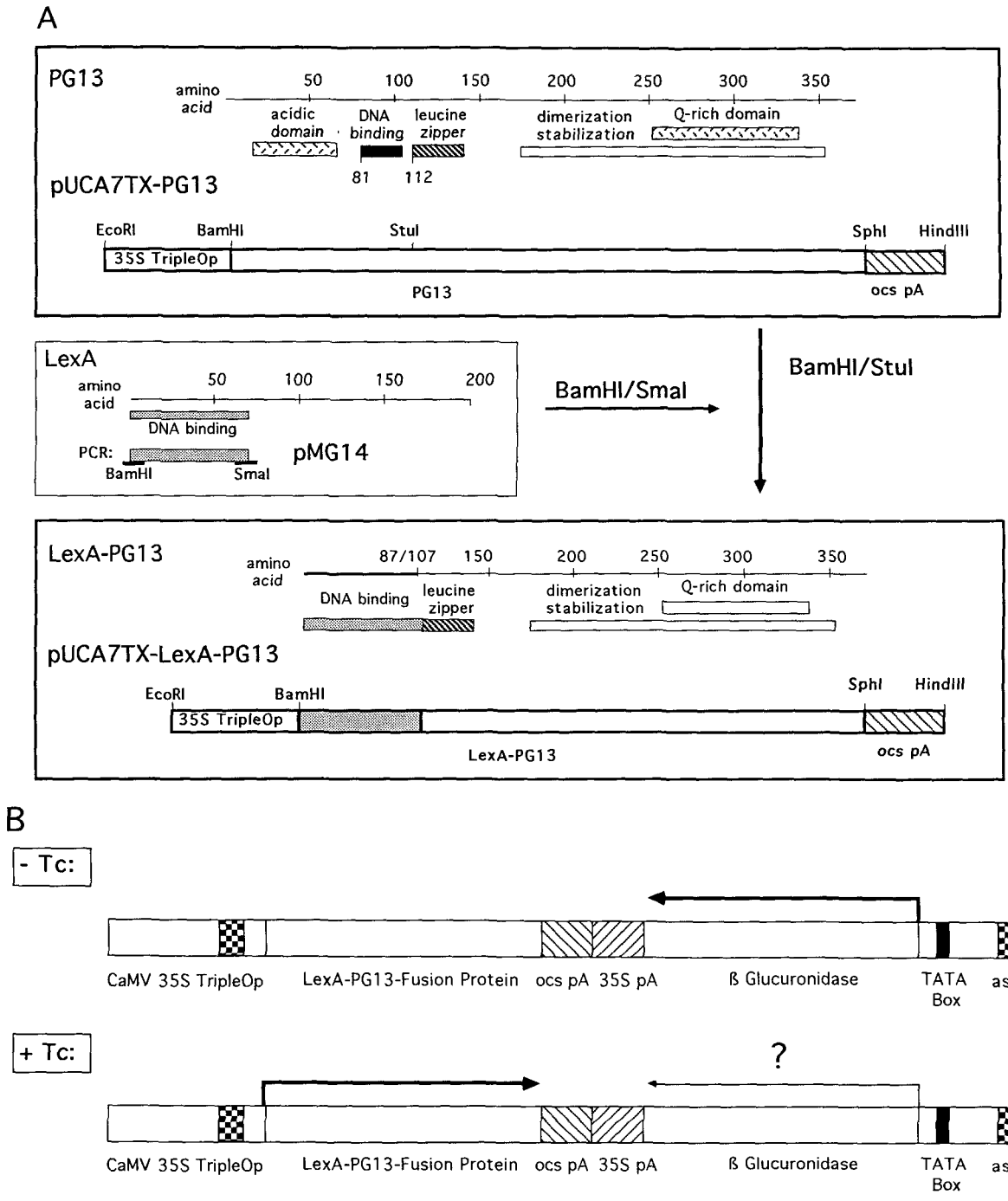


Figure 1. Outline of the Experimental Strategy.

(A) Construction of the *lexA-pg13* gene fusion. Schematic diagrams of proteins PG13, LexA, and LexA-PG13 are shown at the top of each boxed section. Boxes below the amino acid scales indicate the location of the different domains. Restriction sites used to construct pUCA7TX-PG13, pMG14, and pUCA7TX-LexA-PG13 are shown. The BamHI-StuI fragment of pUCA7TX-PG13 was replaced by the BamHI-SmaI PCR fragment derived from pMG14 to exchange amino acids 1 to 107 for amino acids 1 to 87 of the LexA protein. pA, polyadenylation signal; Triple-Op, tetracycline-inducible CaMV 35S promoter.

(B) Arrangement of transgenes on the T-DNA. In the absence of tetracycline (–Tc), transcription of *lexA-pg13* is repressed, whereas the CaMV 35S (–90) promoter is active. By inducing the Triple-Op promoter with tetracycline (+Tc), we investigated whether LexA-PG13 would influence CaMV 35S (–90) promoter activity.

Microinjection experiments with isolated TGA1a protein have shown that high TGA1a levels in leaf cells can mediate transcriptional activation from the CaMV 35S (−90) promoter. Unfortunately, this experiment has only been mentioned as personal communication (G. Neuhaus) in reports published by Katagiri et al. (1992) and Katagiri and Chua (1992) and can therefore not yet be taken as reliable evidence that TGA1a alone can activate *as-1*–dependent transcription in vivo. *tga1a* and *pg13* mRNA levels are higher in roots than in leaves, which correlates with the expression pattern of *as-1*–dependent promoters (Katagiri et al., 1989; Lam et al., 1989; Fromm et al., 1991).

Because the *as-1* motif—either alone or in the context of the complete CaMV 35S promoter—does not mediate activity in leaves (unless they are induced by auxin), and because *tga1a* and *pg13* mRNA levels are low in this tissue, composition and function of the ASF-1 complex in leaf nuclear extracts are still not clear. Mutations in *as-1* that reduce promoter activity also reduce binding of ASF-1 and TGA1a (Katagiri et al., 1989; Lam et al., 1989). This has been taken as strong evidence that TGA1a is at least part of ASF-1.

A function of the TGA1a/PG13 bZIP family and of ASF-1 with regard to expression of endogenous genes has not yet been demonstrated. cDNAs related to *tga1a* have been isolated from wheat (Tabata et al., 1991), Arabidopsis (Kawata et al., 1992; Schindler et al., 1992a; Zhang et al., 1993), maize (Foley et al., 1993), and potato (Feltkamp et al., 1994), implicating a conserved function of these DNA binding proteins in the plant life cycle. In addition, two distinct groups of plant pathogens, Agrobacterium species and DNA viruses, use these conserved host proteins to enhance transcription of their genes. For example, the promoters of the opine synthase genes, such as the octopine synthase (*ocs*; Bouchez et al., 1989; Fromm et al., 1989), nopaline synthase (*nos*; Lam et al., 1990), and mannopine synthase (*mas*; Feltkamp et al., 1994) genes, use the *as-1*–like *cis* element. Recombinant TGA1a, recombinant PG13, and ASF-1 bind to the *as-1*–like elements in these promoters (Bouchez et al., 1989; Fromm et al., 1989; Lam et al., 1990; Feltkamp et al., 1994). Tokuhisa et al. (1990) have exemplarily shown for the ocs element that *as-1*–like elements contain functionally identical tandem nuclear protein binding sites with occupation of both elements being important for transcriptional activation. Except for the CaMV 35S promoter, promoters of the figwort mosaic virus (Bouchez et al., 1989; Cooke, 1990) and the commelina yellow mottle virus (Medberry et al., 1992) encode *as-1*–like elements. Surprisingly, this motif occurs rarely in the plant gene promoters identified thus far (Ellis et al., 1993). The sequence was found in the soybean heat shock gene *gmhsp26-A* and three auxin-responsive tobacco genes (*gnt35* and *gnt1*, van der Zaal et al., 1991; and *par*, Takahashi et al., 1989). One half-site of *as-1* (TGACG) is present in the *hex* motif of the wheat histone H3 gene, but it functions only in combination with an adjacent octamer site that is located 2 bp away (Tabata et al., 1991).

Here, we describe an experimental strategy to decrease the DNA binding activity of the TGA1a/PG13 bZIP family in transgenic plants. In this context, we define the term "family" as a group of bZIP proteins that can interact by forming dimers. We have constructed transgenic tobacco plants expressing a potato *pg13* derivative whose DNA binding domain was replaced by the DNA binding domain of the procaryotic LexA repressor. The resulting chimeric protein LexA-PG13 still retained its ZIP dimerization region as well as the dimer stabilization sequence. The rationale behind this approach was that heterodimers between proteins with interactive ZIP domains but different DNA binding domains do not recognize their target sequences (Ransone et al., 1990). By using an inducible expression system, we avoided counterselecting against those plants that would not regenerate because of a strong decrease of functional TGA1a/PG13 bZIP proteins. The effects of LexA-PG13 on the formation of ASF-1 and on the activity of the CaMV 35S (−90) promoter were studied to define in vivo whether the TGA1a/PG13 bZIP family interacts with proteins involved in these functions.

RESULTS

Outline of the Experimental Strategy

Experiments were performed with a potato cDNA clone, the deduced open reading frame of which shows 91% amino acid identity to PG13 of tobacco (Feltkamp et al., 1994). The basic DNA binding domain is 100% identical to tobacco PG13 and TGA1a. The ZIP domain is 86% identical to the ZIP domain of tobacco PG13 and 76% identical to that of tobacco TGA1a. Because bZIP proteins can form heterodimers even when the ZIP region is only identical in 44% of its positions (Schindler et al., 1992b), we assumed that in transgenic tobacco, domains of potato PG13 would act like the corresponding host domains. The potato *pg13* cDNA contains a convenient StuI restriction site between the deduced basic domain and the ZIP region. As outlined in Figure 1A, this StuI site was used to replace amino acids 1 to 107 by amino acids 1 to 87 of the LexA repressor protein. Because bZIP proteins with mutated basic domains can function as *trans*-dominant negative mutants (Ransone et al., 1990; Unger et al., 1993), it was reasonable to expect that the LexA-PG13 fusion protein might suppress the function of TGA1a, PG13, and related bZIP proteins in vivo. Therefore, in contrast to antisense RNA approaches, the strategy outlined here allowed us to inactivate a whole family of interacting bZIP proteins.

Taking into consideration that inactivation of a whole family of transcription factors might interfere with regeneration, growth, and/or reproduction of resulting transgenic plants, we used the tetracycline-inducible CaMV 35S (Triple-Op) promoter to direct expression of *lexA-pg13* (Gatz et al., 1992). This modified CaMV 35S promoter contains three tetracycline (*tet*) operators in the vicinity of the TATA-box. When transferred into transgenic plants expressing high levels of the Tn*10*-encoded Tet repressor, the activity of this promoter is efficiently shut down. Promoter activity is induced when plants are treated with tetracycline, which prevents binding of the Tet repressor

to the *tet* operator DNA. A β-glucuronidase (*gus*) reporter construct (Jefferson et al., 1987) under the control of the CaMV 35S (−90) promoter was cotransferred with the chimeric *lexA-pg13* gene (Figure 1B). To minimize a potential influence of the full-length CaMV 35S enhancer on expression from the CaMV 35S (−90) promoter, both chimeric genes were arranged in a tail-to-tail orientation. For control experiments, we used plants that contained only the chimeric CaMV 35S (−90):*gus* gene.

LexA-PG13 Interferes with Expression from the CaMV 35S (−90) Promoter

The chimeric genes outlined in Figure 1B were stably integrated into the tobacco genome using *Agrobacterium*-mediated gene transfer (Rosahl et al., 1987). Tobacco (cv Samsun NN) that expresses high levels of the Tet repressor as a result of a previous transformation with pTET1 was used (Gatz et al., 1991a). Half of the explants were regenerated in the presence of tetracycline, the second half in the absence of tetracycline. We did not observe any difference in the efficiency of regeneration.

As shown in Figure 2, RNA gel blot analysis was conducted to analyze expression of the transgenes. Leaf material is a convenient source of RNA. Because the CaMV 35S (−90) promoter is not active in leaves unless they are treated with auxin, we cultivated transgenic plants in the presence of 50 μM 2,4-D for 2 weeks. Expression of *lexA-pg13* was repressed during this period. To ensure efficient induction of *lexA-pg13* expression prior to RNA isolation, we infiltrated single leaves with 1 mg/L tetracycline and incubated them on liquid Murashige and Skoog medium (Murashige and Skoog, 1962) with 2% sucrose (2MS) and 1 mg/L tetracycline for 2 days. RNA from tetracycline-treated and untreated leaves was first hybridized with the *lexA* probe (Figure 2). As expected, plants synthesized *lexA-pg13* mRNA when induced with tetracycline, the only exception being plant E, which showed some expression even in the absence of the inducer. Rehybridization of the blot with a *gus* probe revealed a reciprocal expression pattern. *gus* mRNA was only detectable in the absence of tetracycline (i.e., in the absence of LexA-PG13). Transcription of the *tet* repressor gene under the control of the wild-type CaMV 35S promoter was independent of tetracycline treatment and can thus be taken as a control for loading equal amounts of RNA.

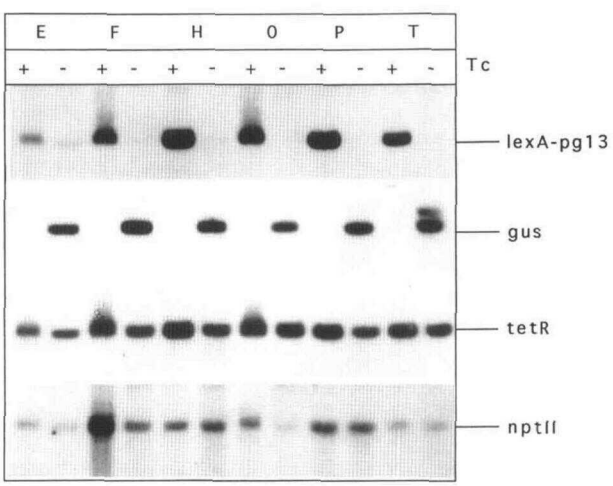


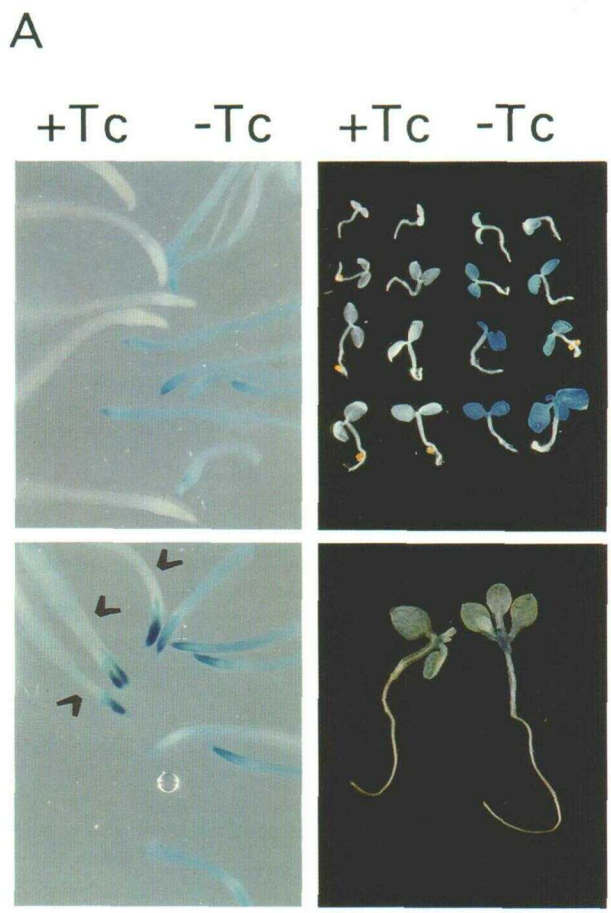
Figure 2. RNA Gel Blot Analysis of Total RNA from Six Independent *lexA-pg13/gus* Plants.

Plants were grown on 2MS with 50 μM 2,4-D. RNA in lanes marked with (+) was extracted from single leaves that were infiltrated with 1 mg/L tetracycline (Tc) and incubated for 48 hr on liquid 2MS and tetracycline. RNA in lanes marked with (−) were taken directly from plants. RNA (30 μg) was loaded in each lane, and hybridization was conducted with the probes indicated at right. tetR, *tet* repressor probe.

We also hybridized the RNA with a neomycin phosphotransferase (*nptII*) probe. The *nptII* gene, which was introduced as a selectable marker along with the *tet* repressor gene (Gatz et al., 1991a), is under the control of the *nos* promoter, whose essential region is recognized by ASF-1 and TGA1a (Katagiri et al., 1989; Lam et al., 1990). Figure 2 clearly shows that in auxin-induced leaves from four of six plants, the *nos* promoter was not affected by LexA-PG13. RNA from plants F and 0 showed an even more intensive hybridization signal in the presence of tetracycline.

Because of the stability of the Gus protein (Weinmann et al., 1994), we did not expect it to disappear within 2 days of induction of *lexA-pg13*. Therefore, we did not perform Gus assays with the material used for RNA analysis. Instead, we compared Gus activities of plants grown on 2MS medium supplemented with 2,4-D or with 2,4-D and tetracycline. Earlier, we had observed that tetracycline induction of whole plants under tissue culture conditions was not as efficient as infiltration experiments with single leaves (Gatz et al., 1992); nevertheless, we were able to detect tetracycline-inducible suppression of *gus* expression under these conditions. Quantitation of Gus activities from independent transformants revealed a three- to 26-fold suppression of gene expression in independent transformants. Control plants that contained the chimeric CaMV 35S (−90):*gus* gene and not the CaMV 35S (Triple-Op):*lexA-pg13* construct showed tetracycline-independent Gus activities.

Ten transgenic *lexA-pg13/gus* plants and control plants (encoding the *gus* gene under the control of the CaMV 35S [−90] promoter and no *lexA-pg13* gene, respectively) were rooted in the presence and absence of tetracycline and stained for Gus activity. Figure 3A (top left) shows the staining pattern of plant H (see also Figure 2). Roots showed Gus activity when grown in the absence of tetracycline. In contrast, *gus* expression in roots from an isogenic cutting grown in the presence of tetracycline was suppressed. Roots from control plants stained whether or not they were grown on tetracycline (bottom left). To assess the effect of LexA-PG13 on wild-type CaMV 35S promoter activity in roots, RNA was extracted from roots of two cuttings of plant H, which had been grown in hydroponic



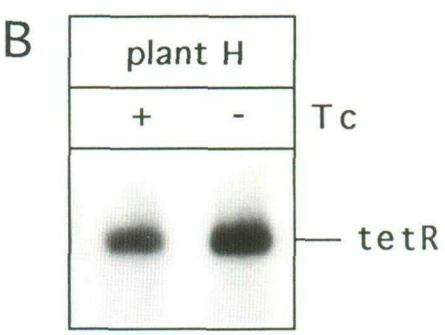


Figure 3. Expression Analysis in Roots and Seedlings.

(A) In situ staining for Gus activities. At top left are roots from *lexA-pg13/gus* transgenic plant H; roots coming in from the left were grown on tetracycline-containing medium (+TC), roots coming in from the right were grown without tetracycline (–TC). At bottom left are roots from a control plant encoding the CaMV 35S (–90):*gus* gene. Roots marked with an arrowhead were grown on tetracycline-containing medium. At top right are seedlings from transgenic plant H (upper part) grown in the presence (+) or absence (–) of tetracycline. Two representative seedlings were stained after 7, 10, 15, and 20 days. At bottom right are seedlings from a control plant encoding the CaMV 35S (–90):*gus* gene grown in the presence or absence of tetracycline at day 20.

(B) RNA gel blot analysis of total RNA from roots. Two cuttings of transgenic plant H were grown in hydroponic solution (with [+] and without [–] tetracycline) for several weeks. Hybridization was conducted with a probe encoding the *tet* repressor gene.

culture with and without tetracycline for several weeks. Hybridization with the *tet* repressor probe revealed no significant decrease in promoter activity (Figure 3B) in tetracycline-treated roots.

Seedlings from one selected plant (plant H, see also Figure 2) and a control plant were germinated in liquid Murashige and Skoog (1962) medium in the presence or absence of tetracycline. Again, the progeny of plant H showed tetracycline-dependent suppression of *gus* expression (Figure 3A, top right), whereas control seedlings showed Gus activity, irrespective of the presence of tetracycline (Figure 3A, bottom right). Gus activity was suppressed by a factor of 20.

Except for roots, auxin-induced leaves, and seedlings, we analyzed whether LexA-PG13 affected growth and gene expression of callus cells. Callus formation was directly induced after Agrobacterium-mediated gene transfer. As observed when regenerating shoots, growth of the transformed tissue was not affected by tetracycline. Individual calli were divided and transferred into liquid medium and grown in the presence or absence of tetracycline. Gus activities were suppressed between three- and 13-fold.

LexA-PG13 Interferes with Formation of ASF-1

ASF-1 is a complex of nuclear proteins that interacts with *as-1* (Katagiri et al., 1989). We performed gel mobility shift assays to test whether ASF-1 formation was affected by LexA-PG13. Two cuttings of *lexA-pg13/gus* plant H and two cuttings of a control plant were cultivated in hydroponic culture. Tetracycline was added to the hydroponic solution of one cutting of each transgenic plant. When plants had six to eight leaves, leaf material was harvested and nuclear proteins were prepared. Figure 4A shows a gel shift experiment of the four extracts with a probe encoding *as-1*. Two specific DNA–protein complexes were observed when binding assays were performed with extracts from tetracycline-treated and untreated control plants and with the extract from the untreated *lexA-pg13/gus* plant H. This gel shift pattern is typical for ASF-1 (Prat et al., 1989). In the faster migrating complex, one TGACG motif is occupied. The second complex runs more slowly because both motifs are bound (Lam et al., 1989). Competition experiments with a 100-fold molar excess of an unlabeled oligonucleotide encoding *as-1* proved the specificity of the complexes (Figure 4A). Proteins able to bind to *as-1* were less abundant in nuclear extracts from tetracycline-treated plant H: the upper band is undetectable; the intensity of the lower band is significantly reduced.

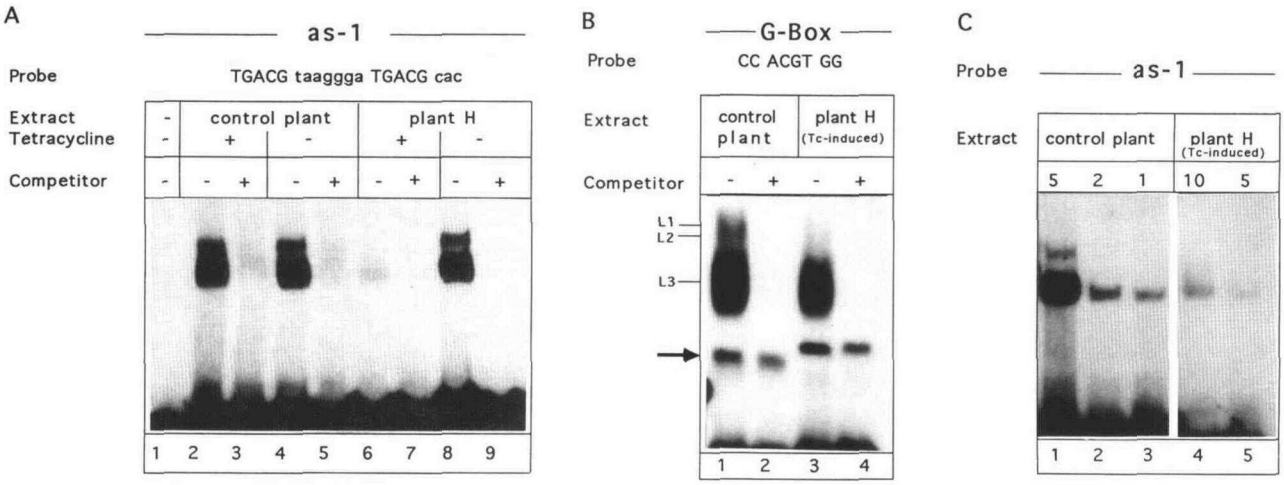


Figure 4. Gel Shift Analysis of Nuclear Extracts.

(A) A radiolabeled 112-bp fragment encoding the *as-1* motif was used as a probe. The binding motif with the two TGACG pentamers is indicated. Nuclear extract (10 μg) was added to binding reactions analyzed in lanes 2 to 9. The control plant does not encode *lexA-pg13*, and plant H encodes *lexA-pg13* under the control of a tetracycline-inducible promoter. Two isogenic cuttings of each plant were grown in the presence of tetracycline (+, lanes 2, 3, 6, and 7) or in the absence of tetracycline (−, lanes 4, 5, 8, and 9). A 100-fold molar excess of unlabeled *as-1*–encoding oligonucleotide was added in lanes 3, 5, 7, and 9. ASF-1 formation is significantly reduced in extracts derived from *lexA-pg13/gus* plant H after tetracycline induction (lane 6). Lane 1 is the probe in the absence of nuclear extracts.

(B) A radiolabeled 30-bp fragment encoding the G-box motif was used as a probe. Nuclear extract (10 μg) was added to binding reactions analyzed in lanes 1 to 4. The arrow points to the unspecific protein–DNA complex; L1, L2, and L3 are different complexes as a result of specific binding of GBFs to the probe. Tc, tetracycline.

(C) A radiolabeled 112-bp fragment encoding the *as-1* motif was incubated with different amounts of extract to roughly calculate how much less ASF-1 is present in tetracycline-induced plant H as compared to control plants. Numbers above the lanes indicate the amount of protein (in micrograms) added to each binding reaction.

To analyze the quality of the extract, a second gel shift assay was performed using the G-box motif as a probe. The G-box (CCACGTGG) is recognized by a class of bZIP G-box binding factors (GBFs) that is distinct from the TGA1a/PG13 bZIP family. TGA1a does not bind to the G-box and does not form heterodimers with the GBFs isolated thus far (Schindler et al., 1992a). Figure 4B demonstrates the result of the experiment. Nuclear proteins from the control plant bound to the G-box probe in a manner similiar to that described for nuclear extracts from tomato (Meier and Gruissem, 1994): a diffuse retarded band (L3), possibly consisting of more than one complex, as well as two minor bands of reduced mobility (L1 and L2) were observed. All three complexes were specifically competed by a 100-fold excess of the unlabeled G-box oligonucleotide. In addition, we observed a sharp, faster migrating complex that did not bind to sequences encoded by the unlabeled competitor DNA. The nuclear extract from the tetracycline-treated *lexA-pg13/gus* plant H contained the proteins giving rise to the unspecific complex as well as to those giving rise to complex L3, proving that the qualities of the extracts were comparable. Interestingly, L1 and L2 were missing, implicating some interaction between LexA-PG13 and the proteins of the L1 and L2 complex.

To determine how much less ASF-1 is formed in tetracycline-treated plant H as compared to control plants, we compared decreasing amounts of nuclear proteins of control extracts with two different concentrations of the extract from tetracycline-treated plant H. Figure 4C illustrates that at least 10-fold less protein is able to bind to *as-1* in tetracycline-treated plant H as compared to control plants.

DISCUSSION

The principal findings of our experiments using transgenic plants with decreased levels of active members of the TGA1a/PG13 bZIP family are fourfold: (1) expression from the CaMV 35S (−90) promoter is reduced; (2) ASF-1 formation is affected; (3) expression from the *nos* promoter is not inhibited; (4) under our conditions, which involved callus induction, regeneration of shoots, growth, flowering, and germination, we did not observe any altered phenotype of the transgenic plants.

Possible Mode of Action of the *trans*-Dominant Negative LexA-PG13 Protein

In this study, we generated and analyzed transgenic tobacco plants expressing a mutant of the potato *as-1* binding protein PG13 (LexA-PG13). LexA-PG13 lacks 107 N-terminal amino

acids comprising the basic DNA binding domain. It retains its ZIP dimerization region as well as the amino acids encoding the dimerization stabilization domain and the Q-rich region (Figure 1A). Because heterodimers between bZIP proteins with different DNA binding domains do not bind to the wild-type target site (Ransone et al., 1990; Unger et al., 1993), an excess of LexA-PG13 was expected to reduce the probability of endogenous related bZIP proteins to form functional dimers with respect to DNA binding. A similar strategy was used by Unger et al. (1993), who deleted most of the basic amino acids adjacent to the ZIP of the maize Opaque2 transcription factor. This mutant inhibited expression from a 22-kD zein:luciferase gene construct in transient expression assays. The authors demonstrated in vitro that mutant and wild-type Opaque2 proteins formed inactive heterodimers unable to bind to their target sequence. We did not directly prove that LexA-PG13 forms heterodimers with TGA1a and PG13 that do not bind to *as-1,* but the specificity of the resulting consequences in the transgenic plants makes this explanation extremely likely. We used the strategy outlined here rather than expressing antisense RNA against endogenous *tga1a* or *pg13* mRNAs, because it allowed us to inactivate a whole family of interacting bZIP proteins, thus enhancing the chances that phenotypic consequences can be observed.

The LexA DNA binding portion of LexA-PG13 was introduced to test whether the LexA-PG13 fusion protein can mediate transcription from a chimeric target promoter containing two *lex* operators upstream of a TATA-box. This type of experiment is generally used (Ptashne, 1988) to prove that a DNA binding protein contains a module responsible for transcriptional activation so that it can be defined as a transcriptional activator. Thus far, we have not observed any transcriptional activation (data not shown). This could be a result of the fact that (1) the protein does not enter the nucleus; (2) the activation domains are not properly positioned (we have also fused the acidic domain to the N terminus of LexA-PG13); (3) a PG13 homodimer cannot activate transcription; or (4) that PG13 is not involved directly in transcriptional activation. Experiments to distinguish between these possibilities are under way.

Advantages of the Tetracycline-Inducible Expression System

One of the general advantages of using an inducible expression system is that plants encoding a gene, which is lethal when expressed strongly, can be regenerated. In retrospect, this precaution was not necessary because plants regenerated equally well under repressed (without tetracycline) or induced (with tetracycline) conditions. In the presence of tetracycline, expression levels of the transgenes in plantlets were equal, whether they had originally been regenerated with or without tetracycline. However, we can state that we did not counterselect against plants expressing levels of *lexA-pg13* that would lead to phenotypic consequences.

The second advantage of the inducible system in this particular experiment is that we can exclude position effects as a cause of any lack of Gus activity. Gus activity is detectable when expression of *lexA-pg13* is repressed, and it is reduced upon tetracycline induction of *lexA-pg13.*

ASF-1 Formation Is Affected upon Induction of *lexA-pg13*

One of the unresolved issues in the understanding of *as-1*–mediated gene expression is the composition and function of ASF-1, a complex of *as-1* binding proteins in nuclear extracts from leaves. The binding specificity of ASF-1 for wild-type and mutant forms of *as-1* suggests that ASF-1 is involved in the in vivo function of *as-1.* To investigate if ASF-1 formation is inhibited by LexA-PG13, we performed gel shift experiments (Figures 4A and 4C). Nuclear extracts from a tetracycline-induced transgenic plant expressing *lexA-pg13* contained at least 10-fold less ASF-1 than uninduced plants or plants that did not encode *lexA-pg13.* This experiment supports the notion that ASF-1 comprises the transcription factors necessary to activate the CaMV 35S (−90) promoter.

There are two possible explanations for how ASF-1 formation might be affected by LexA-PG13. The simplest interpretation is that LexA-PG13 directly forms dimers with the putative bZIP transcription factors of ASF-1. Alternatively, it could be that LexA-PG13 might inactivate factors that could be involved in transcription of genes encoding ASF-1 proteins. Mixing extracts from tetracycline-treated plant H and control plants did not help to distinguish between these possibilities: the extract from tetracycline-treated plant H did not reduce the amount of ASF-1 in the extract of the control plant (data not shown). It could either be that the exchange of monomers is not efficient enough in vitro or that LexA-PG13 is primarily located in the cytoplasm and, thus, is lost during the preparation. Considering that promoters encoding *as-1* among other *cis*-acting sequences (like the wild-type CaMV 35S promoter and the *nos* promoter, see below) are not affected by LexA-PG13, we favor the notion that ASF-1 formation is directly inhibited, rather than that transcription of corresponding genes is reduced.

The arrangement of transgenes outlined in Figure 1B might raise the argument that suppression of transcription of the *gus* gene upon induction of the Triple-Op promoter might be a result of the synthesis of an RNA complementary to the *gus* mRNA because of inefficient transcriptional termination. The result of the gel retardation experiments strongly argues against this possibility. In addition, transcriptional termination should not be so inefficient that enough *gus* antisense RNA is synthesized. Moreover, antisense effects only work in a fraction of plants (van der Krol et al., 1988), whereas we observe a negative effect on *gus* expression in all our hygromycin-resistant plants.

If we assume that the residual binding activity in nuclear extracts of LexA-PG13–expressing plant H is the result of a dimer of wild-type bZIP proteins, rather than to weak binding of a wild-type/mutant heterodimer, the excess of LexA-PG13 was not high enough to completely titrate out all endogenous TGA1a/PG13 bZIP proteins. It could also be that dimerization

between the potato protein and the endogenous tobacco proteins was too inefficient to allow complete reduction of the ASF-1 complex. Because we regenerated shoots and callus in the absence of the *trans*-dominant negative protein, we can rule out the possibility that we have selected against plants with higher expression levels.

Alternatively, it could well be that the residual band is a result of a second family of transcriptional activators that binds to *as-1* but does not heterodimerize with the TGA1a/PG13 bZIP family. The incomplete suppression of ASF-1 formation in leaves correlates with the observation that Gus activity was also not totally suppressed in the tissues where *as-1* is active. We generally observed a three- to 26-fold inhibition of Gus activities in independent transgenic plants when these plants were treated with tetracycline.

Gel retardation experiments using the G-box as a probe served two purposes. First, we wanted to ensure that the reduced amount of ASF-1 was not the result of degradation of nuclear proteins or a general inhibitor of DNA binding activities in that specific nuclear extract. Second, we asked the question of whether bZIP proteins with different binding specificities were also affected. G-boxes are recognized by a subset of bZIP proteins called GBFs (Oeda et al., 1991; Weisshaar et al., 1991; Schindler et al., 1992b). Three recombinant Arabidopsis GBFs do not heterodimerize with the TGA1a/PG13 family in vitro (Schindler et al., 1992a). Our tobacco leaf extracts gave rise to specific and unspecific complexes (Figure 4B). The unspecific complex, which has also been observed by others (Oeda et al., 1991; Schindler et al., 1992b), does not bind to the unlabeled competitor DNA. It is also not affected in the extract of tetracycline-induced plant H, proving that the extract contains functional DNA binding proteins. The extract also showed a G-box–specific diffuse retarded band (L3), which resembles the complexes observed by Oeda et al. (1991) and Schindler et al. (1992b) in tobacco extracts. It is not known whether the different protein–DNA complexes are the result of different post-translational modifications or multiple GBF-like DNA binding proteins. In addition, we observed two specific complexes of lower mobility (L1 and L2), which have also been observed in tomato nuclear extracts (Meier and Gruissem, 1994). L1 and L2 can be competed by a 100-fold excess of G-box oligonucleotide in tobacco extracts, whereas Meier and Gruissem (1994) described a requirement of higher concentrations for efficient competition. Formation of L3 is not affected by LexA-PG13, demonstrating that (1) the extract contained functional DNA binding proteins, and (2) LexA-PG13 does not generally interfere with DNA binding of bZIP proteins. Interestingly, L1 and L2 were not formed in the presence of LexA-PG13, indicating some interaction of the TGA1a/PG13 bZIP family with as yet uncharacterized members of the GBF family.

Expression from the CaMV 35S (−90) Promoter Is Reduced upon Induction of *lexA-pg13*

Tobacco cDNA clones of the TGA1a/PG13 family have been isolated because of their affinity to *as-1* (Katagiri et al., 1989; Fromm et al., 1991). To test whether expression of a *trans*-dominant negative mutant of *pg13* affected *as-1*–dependent gene expression, a stably integrated CaMV 35S (−90):*gus* gene was used as a reporter construct.

We observed tetracycline-inducible suppression of *gus* expression in auxin-induced leaves (Figure 2), roots (Figure 3A), seedlings (Figure 3A), and callus cells. Our results show that the TGA1a/PG13 bZIP family forms dimers with proteins involved either directly or indirectly in the regulation of expression from the CaMV 35S (−90) promoter in vivo. However, our experiments do not prove that TGA1a or PG13 are bona fide transcriptional activators. It has been shown in vitro that TGA1a is able to activate transcription from a promoter that contains two *as-1* sites upstream of a TATA-box (Katagiri et al., 1990; Yamazaki et al., 1990). This is not equivalent to the situation analyzed in vivo, where one *as-1* site is sufficient for activation. Data on the potential of TGA1a to activate transcription in vivo have only been mentioned by Katagiri et al. as personal communications (G. Neuhaus) in original papers (Katagiri et al., 1992) or review articles (Katagiri and Chua, 1992).

LexA-PG13 Does Not Suppress *nos* Promoter Activity

Transgenic plants were also used for an initial analysis to test whether *as-1*–dependent gene expression is also affected when *as-1* is part of a larger promoter context. It has been reported that ASF-1 binds to an essential region of the *nos* promoter, implying that the same family of factors regulates expression from *as-1* and from the *nos* promoter (Lam et al., 1990). The respective binding motif of the *nos* promoter differs from the *as-1* site: it contains a TGACG motif in the inverse orientation and a TGAGC motif instead of two tandemly repeated TGACG motifs. The *nptII* gene is driven by the *nos* promoter in our transgenic plants (Gatz et al., 1991a). Therefore, we reprobed the RNA gel blot with the *nptII* fragment. Unexpectedly, *nos* promoter activity in auxin-induced leaves was not negatively affected by LexA-PG13 (Figure 2). It could therefore be that there is a second class of factors that does not form heterodimers with the TGA1a/PG13 bZIP family but competes for the similar binding motif. The enhancement of *nos* promoter activity in two of six plants upon *lexA-pg13* expression might be the result of better access of the second family to the promoter region in the absence of functional ASF-1.

Alternatively, it could well be that the affinity of plant transcription factors to the *as-1*–like element within the complete *nos* promoter is higher as compared to the isolated *as-1* element of the CaMV 35S (−90) promoter. In the wild-type promoter context, increased binding affinities to a promoter region might be established by protein–protein interactions with transcription factors bound to sequences flanking the element. Deletion analysis of the *nos* promoter has shown that the *as-1*–like element is crucial for promoter activity, but that flanking sequences enhance transcriptional activation (Ebert et al., 1987). Because the *trans*-dominant negative LexA-PG13 protein does not completely titrate away all the proteins able to

bind to *as-1* (see above), the residual amounts of activators might be sufficient to sustain full *nos* promoter activity.

This might also explain why the wild-type CaMV 35S promoter is not affected in auxin-induced leaves, where it has been shown at the level of RNA that *as-1* contributes to the activity of the full-length promoter (Liu and Lam, 1994). Using the *gus* reporter system, Benfey et al. (1989) have demonstrated that *as-1* is necessary to mediate maximal expression of the CaMV 35S promoter in roots. At the mRNA level, we detected only a slight suppression of transcription by LexA-PG13 in roots (Figure 3B), which raises the question of whether *as-1*–mediated gene expression in a normal promoter context can be suppressed by the approach outlined here. Using the *gus* reporter system, which allows detection of expression patterns in different cell types, synergistic interaction between *as-1* and sequences located further upstream has been observed at specific stages of the developing seedling. Careful analysis of a CaMV 35S:*gus* construct introduced into plants expressing *lexA-pg13* will reveal to what extent *trans*-dominant suppression affects synergistic interactions between *as-1* and other *cis* elements in the context of the full-length promoter.

Phenotype of Transgenic Plants

Because of the conservation of the TGA1a/PG13 bZIP family in different plant species, the relative abundance of ASF-1 in leaves, and the use of these factors by independent plant pathogens, we considered it likely that inactivation of the whole family of these transcription factors might affect the phenotype of the plant. However, under our conditions, which involved callus induction, regeneration of shoots, growth, flowering, and germination, we did not observe any negative effect when we lowered ASF-1 abundance by a factor of at least 10, which was sufficient to lower CaMV 35S (−90) promoter activity by a factor of up to 26.

We have discussed above why the *nos* promoter might not be affected. The same arguments (incomplete *trans*-dominant suppression, independent transcriptional activators) might hold for endogenous plant promoters that contain TGACG motifs. Alternatively, it could also be that we have not yet found the conditions where high levels of members of the TGA1a/bZIP family are important for survival of the plant.

Conclusion

We have constructed transgenic plants encoding a *trans*-dominant negative transcription factor under the control of an inducible expression system. We were able to show that the dimerization domains of PG13 interact with proteins involved in *as-1*–mediated transcription and ASF-1 formation. Proteins involved in transcription from the *nos* promoter were not inactivated, raising the question of whether the same family of factors regulates the CaMV 35S (−90) promoter and the *nos* promoter. These transgenic plants will be used in the future to analyze the function of the TGA1a/PG13 bZIP family on promoters containing *as-1*–like elements in the context of multiple regulatory elements.

METHODS

Plants, Bacterial Strains, and Media

Nicotiana tabacum cv Samsun NN was obtained through Vereinigte Saatzuchten (Ebstorf, Germany). Plants in tissue culture were grown under a 16-hr-light/8-hr-dark regime on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% sucrose (2MS). *Escherichia coli* DH5α (Bethesda Research Laboratories) was cultivated using standard techniques (Sambrook et al., 1989). *Agrobacterium tumefaciens* C58CX1 containing pGV2260 (Deblaere et al., 1985) was cultivated in YEB medium (Vervliet et al., 1975).

Recombinant DNA Techniques

Standard procedures were used for recombinant DNA analysis (Sambrook et al., 1989).

Constructs

The potato *pg13* cDNA encoding 140 bp of the 5′ untranslated sequence and 240 bp of the 3′ untranslated sequence was cloned into the HincII site of pUC18 with the N-terminal sequence oriented toward the BamHI site of the polylinker. The fragment was excised with BamHI and SpHI and cloned into pUCA7TX. pUCA7TX contains an expression cassette that allows different coding regions to be placed between the tetracycline-inducible cauliflower mosaic virus (CaMV) 35S (Triple-Op) promoter (Gatz et al., 1992) and the octopine synthase (*ocs*) polyadenylation signal. The resulting plasmid pUCA7TX-PG13 (Figure 1A) was cut with BamHI and StuI to excise the 5′ untranslated region as well as the first 107 amino acids. The *lexA* DNA binding region (corresponding to amino acids 1 to 87) was amplified from pMG14 (Oertelt-Buchheit et al., 1990) using polymerase chain reaction (PCR). The N-terminal primer (GG-*GGATCC*-GTAGACCC-ATGAAAGCGTTAACGGCCAG) encodes the first six codons (underlined) as well as a BamHI (printed in italics) restriction site. The C-terminal primer (GCTCTAGA-*CCCGG* GGTTCACCGGCAGCCACACGACC) encodes amino acids 80 to 87 (underlined) as well as an SmaI site (printed in italics). The PCR product was cut with BamHI and SmaI and cloned into pUCA7TX-PG13 cut with BamHI and StuI. The PCR-generated region as well as the LexA/PG13 junction was sequenced (Sanger et al., 1977).

The chimeric gene including the promoter and polyadenylation site was inserted as an EcoRI-HindIII fragment into the binary vector BIN-Hyg (Becker, 1990), resulting in pLexA-PG13. To construct the reporter gene, pLUP+11, which contains the CaMV 35S (−90) promoter upstream of the chloramphenicol acetyltransferase (*cat*) coding region (Frohberg et al., 1991), was cut with BglII and HindIII, which released the coding region. A modified β-glucuronidase (*gus*) gene containing an intron and the CaMV 35S polyadenylation signal (Vancanneyt et al., 1990) was placed as a BamHI-HindIII fragment downstream of the promoter. To construct pLexA-PG13/Gus, the chimeric *gus* gene was cut out with EcoRI and HindIII, ends were made blunt using the Klenow fragment of *E. coli* DNA polymerase I, and they were inserted into pLexA-PG13 cut with HindIII (fill in).

Binding Studies with Nuclear Extracts

Preparation of nuclear extracts was done as described previously (Prat et al., 1989). For gel shift experiments using activating sequence-1 (*as-1*) as a probe, the CaMV 35S (−90) promoter was cut out from pIGF107(−B). pIGF107(−B) is a derivative of pIGF107 (Gatz et al., 1991b), which contains a deletion of promoter sequences upstream of the EcoRV site. A BglII/EcoRI digestion resulted in a 112-bp fragment containing *as-1* as well as the TATA-box of the CaMV 35S promoter. The CaMV 35S (−90) derivative of pIGF107(−B) contains several base pair exchanges around the TATA-box. The sequence of the probe is

AATTCGAGCTCGGTACCCATCCCACT**GACGTAAGGGATGACGCA**
CAATCCCACTAGTCTTCGCAAGACCCTTTACG**TATATAA**GGCCT
TTCTAGACATTTGCTCGAGATC

Base pair exchanges deviating from the wild-type CaMV 35S promoter are underlined. *as-1* and TATA-box are shown in boldface letters. The EcoRI-BglII fragment was radiolabeled by filling in the 5′ overhangs with α-^{32}P-dATP and α-^{32}P-dCTP with the Klenow fragment and gel purified. As competitor, DNA-annealed oligonucleotides were used. The sequence is

5′ GATATCTCCAC**TGACGTAAGGGATGACG**TTAAC

Binding reactions were done as described in Prat et al. (1989). The probe encoding the G-box was cut out of a pUC vector obtained from B. Weisshaar (Max Planck Institut für Züchtungsforschung, Cologne, Germany) using BglII and BamHI. The sequence is

5′ GATCTCTTATT**CCACGTGG**CCATCCGGATC

The G-box is shown in boldface letters. As competitor, annealed oligonucleotides containing the same sequence were used. Labeling and purification of the probe were done as described above.

RNA Gel Blot Analysis

Total RNA from leaves was prepared according to Logemann et al. (1987). Blotting and hybridization was performed as described by Heyer and Gatz (1992).

Assays for Gus Activity

For the fluorometric Gus assay, explants were homogenized and incubated with the substrate 4-methylumbelliferyl β-D-glucuronide at 37°C. Quantification of the fluorescence was done according to Jefferson (1987) and Jefferson et al. (1987). Protein concentrations were determined according to Bradford (1979). For in vivo staining of roots and seedlings, intact plant material was incubated in 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid cyclohexylammonium) overnight at 37°C.

Tobacco Transformation

Transformation of tobacco plants was performed using the *A. tumefaciens* leaf disc technique as described by Rosahl et al. (1987).

Application of Tetracycline to Plants

For tetracycline induction under axenic conditions, plants were grown on 2MS medium with 1 mg/mL tetracycline. Single leaves were infiltrated with tetracycline (Gatz et al., 1991a). Plants used for the preparation of nuclear extracts were cultivated in a beaker containing Hoagland buffer and 1 mg/L tetracycline, which was changed every other day. Oxygen was supplied through an aquarium pump (Gatz et al., 1992).

ACKNOWLEDGMENTS

We would like to thank Drs. Manfred Schnarr (Strasbourg) for providing pMG14, Rainer Höfgen (Berlin) for providing pUCA7TX and the transgenic tobacco cultivar Samsun NN expressing high levels of the Tet repressor, Bernd Weisshaar (Cologne) for the G-box plasmid and for improving the manuscript, and Sabine Rosahl (Cologne) for communicating results prior to publication. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (No. Ga330/3-1).

Received April 6, 1994; accepted June 23, 1994.

REFERENCES

Becker, D. (1990). Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucl. Acids Res. **18,** 203.

Benfey, P.N., and Chua, N.-H. (1990). The cauliflower mosaic virus 35S promoter: Combinatorial regulation of transcription in plants. Science **250,** 959–966.

Benfey, P.N., Ren, L., and Chua, N.-H. *(1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns.* EMBO J. **8,** 2195–2202.

Bouchez, D., Tokuhisa, J.G., Llewellyn, D.J., Dennis, E.S., and Ellis, J.G. (1989). The ocs-element is a component of the promoters of several T-DNA and plant viral genes. EMBO J. **8,** 4197–4204.

Bradford, M.M. (1979). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72,** 248–254.

Cooke, R. (1990). The figwort mosaic virus gene VI promoter region contains a sequence highly homologous to the octopine synthase (*ocs*) enhancer element. Plant Mol. Biol. **15,** 181–187.

Deblaere, R., Bytebier, B., De Greve, H., Debroeck, F., Schell, J., van Montagu, M., and Leemans, J. *(1985). Efficient octopine Ti plasmid derived vectors for Agrobacterium-mediated gene transfer to plants.* Nucl. Acids Res. **13,** 4777–4788.

Ebert, P.R., Ha, S.B., and An, G. (1987). Identification of an essential upstream element in the nopaline synthase promoter by stable and transient assays. Proc. Natl. Acad. Sci. USA **84,** 5745–5749.

Ellis, J.G., Tokuhisa, J.G., Llewellyn, D.J., Bouchez, D., Singh, K., Dennis, E.S., and Peacock, W.J. (1993). Does the ocs-element occur as a functional component of the promoters of plant genes? Plant J. **4,** 433–443.

Feltkamp, D., Masterson, R., Starke, J., and Rosahl, S. (1994). Analysis of the involvement of *ocs*-like bZip-binding elements in the differential strength of the bidirectional *mas*1'2' promoter. Plant Physiol. **105,** 259–268.

Foley, R.C., Grossmann, C., Ellis J.G., Llewellyn, D.J., Dennis, E.S., Peacock, W.J., and Singh K.B. (1993). Isolation of a maize bZIP protein subfamily: Candidates for the ocs-element transcription factor. Plant J. **3,** 669–679.

Frohberg, C., Heins, L., and Gatz, C. (1991). Characterization of the interaction of plant transcription factors using a bacterial repressor protein. Proc. Natl. Acad. Sci. USA **88,** 10470–10474.

Fromm, H., Katagiri, F., and Chua, N.-H. (1989). An octopine synthase enhancer element directs tissue-specific expression and binds ASF-1, a factor from tobacco nuclear extracts. Plant Cell **1,** 977–984.

Fromm, H., Katagiri, F., and Chua, N.-H. (1991). The tobacco transcription activator TGA1a binds to a sequence in the 5' upstream region of a gene encoding a TGA1a-related protein. Mol. Gen. Genet. **229,** 181–188.

Gatz, C., Kaiser, A., and Wendenburg, R. (1991a). Regulation of a modified CaMV 35S promoter by the Tn*10*-encoded Tet repressor in transgenic tobacco. Mol. Gen. Genet. **227,** 229–237.

Gatz, C., Katzek, J., Prat, S., and Heyer, A. (1991b). Repression of the CaMV 35S promoter by the octopine synthase enhancer element. FEBS Lett. **293,** 175–178.

Gatz, C., Frohberg, C., and Wendenburg, R. (1992). Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. Plant J. **2,** 397–404.

Heyer, A., and Gatz, C. (1992). Isolation and characterization of a cDNA-clone coding for potato type A phytochrome. Plant Mol. Biol. **18,** 535–544.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Katagiri, R., and Chua, N.-H. (1992). Plant transcription factors: Present knowledge and future challenges. Trends Genet. **8,** 22–31.

Katagiri, F., Lam, E., and Chua, N.-H. (1989). Two tobacco DNA-binding proteins with homology to nuclear factor CREB. Nature **340,** 727–730.

Katagiri, F., Yamazaki, K.-I., Horikoshi, M., Roeder, R.G., and Chua, N.-H. (1990). A plant DNA-binding protein increases the number of active preinitiation complexes in a human *in vitro* transcription system. Genes Dev. **4,** 1899–1909.

Katagiri, F., Seipel, K., and Chua, N.-H. (1992). Identification of a novel dimer stabilization region in a plant bZIP transcriptional activator. Mol. Cell. Biol. **12,** 4809–4816.

Kawata, T., Imada, T., Shiraishi, H., Okada, K., Shimura, Y., and Ivabuchi, M. (1992). A cDNA clone encoding an HBP-1b homologue in *Arabidopsis thaliana*. Nucl. Acids Res. **20,** 1141–1147.

Lam, E., Benfey, P.N., Gilmartin, P.M., Fang, R.-X., and Chua, N.-H.(1989). Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. Proc. Natl. Acad. Sci. USA **86,** 7890–7894.

Lam, E., Katagiri, F., and Chua, N.-H. (1990). Plant nuclear factor ASF-1 binds to an essential region of the nopaline synthase promoter. J. Biol. Chem. **265,** 9909–9913.

Lamb, P., and McKnight, S.L. (1991). Diversity and specificity in transcriptional regulation: The benefits of heterotypic dimerization. Trends Biochem. Sci. **16,** 417–422.

Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988). The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science **240,** 1759–1764.

Liu, X., and Lam, E. (1994). Two binding sites for the plant transcription factor ASF-1 can respond to auxin treatments in transgenic tobacco. J. Biol. Chem. **269,** 668–675.

Logemann, J., Schell, J., and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. Anal. Biochem. **163,** 16–20.

McKnight, S.L. (1991). Molecular zippers in gene regulation. Sci. Am. **264,** 54–64.

Medberry, S.L., Lockhart, B.E.L., and Olszewski, N.E. (1992). The commelina yellow mottle virus promoter is a strong promoter in vascular and reproductive tissues. Plant Cell **4,** 185–192.

Meier, I., and Gruissem, W. (1994). Novel conserved sequence motifs in plant G-box binding proteins and implications for interactive domains. Nucl. Acids Res. **22,** 470–478.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Oeda, K., Salinas, J., and Chua, N.-H. (1991). A tobacco bZIP transcription activator (TAF-1) binds to a G-box like motif conserved in plant genes. EMBO J. **10,** 1793–1802.

Oertelt-Buchheit, P., Lamerichs, R.M.J.N., Schnarr, M., and Granger-Schnarr, M. (1990). Genetic analysis of the LexA repressor: Isolation and characterization of LexA (Def) mutant proteins. Mol. Gen. Genet. **223,** 40–48.

Prat, S., Willmitzer, L., and Sanchez-Serrano, J.J. (1989). Nuclear proteins binding to a cauliflower mosaic virus 35S truncated promoter. Mol. Gen. Genet. **17,** 209–214.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. Nature **335,** 683–689.

Ransone, L.J., Visvader, J., Wamsley, P., and Verma, I.M. (1990). Trans-dominant negative mutants of Fos and Jun. Proc. Natl. Acad. Sci. USA **87,** 3806–3810.

Rosahl, S., Schmidt, R., Schell, J., and Willmitzer, L. (1987). Expression of a tuber-specific storage protein in transgenic tobacco plants: Demonstration of an esterase activity. EMBO J. **6,** 1155–1159.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schindler U., Beckmann, H., and Cashmore, A.R. (1992a). TGA1 and G-box binding factors: Two distinct classes of Arabidopsis leucine zipper proteins compete for the G-box–like element TGACGTGG. Plant Cell **4,** 1309–1319.

Schindler, U., Menkens, A.E., Beckmann, H., Ecker, R.E., and Cashmore, A.R. (1992b). Heterodimerization between light-regulated and ubiquitously expressed *Arabidopsis* GBF bZIP proteins. EMBO J. **11,** 1261–1273.

Tabata, T., Nakayama, T., Mikami, K., and Iwabuchi, M. (1991). HBP-1a and HBP-1b: Leucine zipper type transcription factors of wheat. EMBO J. **10,** 1459–1467.

Takahashi, Y., Kuroda, H., Tanaka, R., Machida, T., and Nagata, T. (1989). Isolation of an auxin-regulated gene: cDNA Expressed during transition from G0 to S phase in tobacco mesophyll protoplasts. Proc. Natl. Acad. Sci. USA **86,** 9279–9283.

Tokuhisa, J.G., Singh, K., Dennis, E.S., and Peacock, W.J. (1990). A DNA-binding protein factor recognizes two binding domains within the octopine synthase enhancer element. Plant Cell **2,** 215–224.

Unger, E., Parsons, R.L., Schmidt, R.J., Bowen, B., and Roth, B.A. (1993). Dominant negative mutants of Opaque2 suppress transactivation of a 22-kD zein promoter by Opaque2 in maize endosperm cells. Plant Cell **5,** 831–841.

Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L., and Rocha-Sosa, M. (1990). Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol. Gen. Genet. **220,** 245–250.

van der Krol, A.R., Lenting, P.E., Veenstra, J., van der Meer, I.M., Koes, R.E., Gerats, A.G.M., Mol, J.N.M., and Stuitje, A.R. (1988). An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature **333,** 866–869.

van der Zaal, E.J., Droog, F.N.J., Boot, C.J.M., Hensgens, L.A.M., Hoge, J.H.C., Schilperoort, R.A., and Libbenga, K.R. (1991). Promoters of auxin-induced genes from tobacco can lead to auxin-inducible and root tip-specific expression. Plant Mol. Biol. **10,** 145–157.

Vervliet, G., Holsters, M., Teuchy, H., van Montagu, M., and Schell, J. (1975). Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. J. Gen. Virol. **26,** 33–48.

Weinmann, P., Gossen, M., Hillen, W., Bujard, H., and Gatz, C. (1994). A chimeric transactivator allows tetracycline-responsive gene expression in whole plants. Plant J. **5,** 559–569.

Weisshaar, B., Armstrong, G.A., Block, A., da Costa e Silva, O., and Hahlbrock, K. (1991). Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J. **10,** 1777–1786.

Yamazaki, K.-I., Katagiri, F., Imaseki, H., and Chua, N.-H. (1990). TGA1a, a tobacco DNA-binding protein, increases the rate of initiation in a plant *in vitro* transcription system. Proc. Natl. Acad. Sci. USA **87,** 7035–7039.

Zhang, B., Foley, R.C., and Singh, K.B. (1993). Isolation and characterization of two related *Arabidopsis* ocs-element bZIP binding proteins. Plant J. **4,** 711–716.