Regulation of transgene expression in plants with polydactyl zinc finger transcription factors

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Contributed by Roger N. Beachy, August 6, 2002

Designer zinc finger transcription factors (TFs_{ZF}) have been developed to control the expression of transgenes and endogenous genes in mammalian cells. Application of TFs_{ZF} technology in plants would enable a wide range of both basic and applied studies. In this paper, we report the use of TFs_{ZF} to target a defined 18-bp DNA sequence to control gene expression in plant cells and in transgenic plants. A β -glucuronidase reporter gene was activated by using the designed six-zinc finger protein 2C7 expressed as a fusion with the herpes simplex virus VP16 transcription factor activation domain. Reporter gene expression was activated 5- to 30-fold by using TFs_{ZF} in BY-2 protoplasts, whereas expression was increased as much as 450 times in transgenic tobacco plants. Use of a phloem-specific promoter to drive expression of the TFs_{ZF} resulted in activation of the reporter gene in vascular tissues. Transgenic tobacco plants that produce 2C7 transcription factors were phenotypically normal through two generations, suggesting that the factors exerted no adverse effects. This study demonstrates the utility of zinc finger technology in plants, setting the stage for its application in basic and applied agricultural biotechnology.

Contemporary models of eukaryotic gene expression recognize the dynamic mechanisms and pathways that, in the end, determine whether or not a gene is expressed and to what level. Mechanisms that regulate gene expression include chromatin modification and remodeling, binding of multiple factors to DNA cis elements, binding of the RNA polymerase, transcription elongation, termination, and posttranscriptional regulation. The interactions between protein factors and DNA sequences at or near the gene promoter are key components in gene regulation and depend on the accessibility of the region in the context of chromatin (1).

A variety of technical approaches have been used to control gene expression experimentally. One common approach involves the use of synthetic or chimeric genes and transcription factors (2). For example, the yeast galactose inducible Gal4 DNA-binding protein has been fused with activation or repression domains to create previously uncharacterized transcription factors that can act on target genes that incorporate the Gal4 operator sequence (3–5). Recently, synthetic transcription factors based on the assembly of multiple zinc finger domains have been developed for similar purposes. Zinc finger transcription factors are among the most common transcription factors and there may be as many as 700 genes that encode such factors in the human genome. It is estimated that *Arabidopsis* contain 85 genes that encode zinc finger transcription factors (6). Such synthetic zinc fingers transcription factors (TFs_{ZF}) can be custom designed for binding to any DNA sequence (7, 8).

A single zinc finger typically binds three nucleotides, and “polydactyl” fingers comprising multiple zinc fingers have been developed to bind unique DNA sequences and to regulate gene expression (7, 8). Six-finger proteins bind 18 contiguous nucleotides, are predicted to be highly specific for the sites to which they bind, and can potentially be used to regulate expression of genes in many types of organisms. A number of studies have shown that TFs_{ZF} can activate or repress the expression of transgenes or endogenous genes in mammalian cells (7–11).

We initiated the study described herein to determine whether TFs_{ZF} can be used to regulate gene expression in plant cells. We used a well characterized polydactyl six-zinc finger protein, referred to as 2C7, and its cognate 18-bp 2C7 DNA-binding sequence (11), in plant protoplasts and in transgenic plants. In these studies we found that binding of the 2C7 protein increased the expression of three different core promoters. Furthermore, expression in both protoplasts and transgenic plants increased when the herpes simplex virus VP16 activation domain was added to the zinc finger protein. In transgenic plants that contain the zinc finger protein and a reporter gene construct, we observed a >450-fold induction of gene expression. These studies support the conclusion that TFs_{ZF} can be a powerful tool to regulate gene expression in plants and will be useful both in plant genomics and plant biotechnology.

Experimental Procedures

Construction of Plasmids. Promoter fragments from Cassava Vein Mosaic Virus (CsVMV) (12) designated as D (nucleotides –178 to +72), E (nucleotides –112 to +72), and F (nucleotides –63 to +72) were subcloned in the pBluescript II KS(+) vector (Stratagene) and fused to the *uidA* gene (pILTAB402, pILTAB403, and pILTAB404, respectively). Plasmids C7D::G, C7E::G, and C7F::G were constructed by inserting six copies of the 2C7 recognition site from the previously described pGL3 promoter construct (11) via restriction sites *Mlu*I and *Bgl*II. These sites are 5' of the D, E, and F fragments of the CsVMV promoter between *Xba*I and *Bam*HI restriction sites. All gene constructs were confirmed by DNA sequencing (Fig. 1A). The T fragment (nucleotides –32 to +45) of the promoter from Rice Tungro Bacilliform virus (RTBV) was ligated to six tandem repeats of the 2C7-binding sites into the *Xba*I site upstream of the fragment (C7T::G). The T fragment contains the TATA box of RTBV promoter plus nucleotides to +45 (ref. 13; Fig. 2A). Plasmid C7er2::G contains the minimal promoter of the *erbB-2* gene (9). In this plasmid the *Luc* gene was replaced by the *uidA* gene with the nos terminator sequence by using the restriction sites *Nco*I and *Kpn*I (Fig. 2A).

To obtain the expression plasmids that produce the effector proteins (TFs_{ZF}), the ORF encoding the polydactyl protein 2C7 (11), with the transcription activator domain VP16 (11) or VP64 (9) were excised with *Bam*HI and *Hind*III from pcDNA3 (11). The fragment was subcloned in *Bgl*II and *Eco*RI sites of a plasmid, which contains the enhanced 35S promoter from cauliflower mosaic virus (e-35S) promoter (14) and the nos terminator sequences (Figs. 1A and 2A).

To produce the reporter gene for transgenic plants, we subcloned the C7F::G construct (Fig. 3A), with *Not*I and *Hind*III, into the *Bam*HI site of pGJ3571. The TFs_{ZF} constructs were removed from their respective plasmids with *Not*I and placed in

Abbreviations: GUS, β -glucuronidase; CsVMV, Cassava Vein Mosaic virus; RTBV, Rice Tungro Bacilliform virus; TFs_{ZF}, zinc finger transcription factor; e-35S, enhanced 35S promoter; MU, 4-methylumbelliferone.

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Fig. 1. Regulation of GUS expression in BY-2 protoplasts using fragments of the CsVMV promoter with and without the TF_{5ZF} effector genes. (A) Diagram of the reporter and effector constructs. Different lengths of the CsVMV promoter, D (nucleotides -178 to +72), E (nucleotides -112 to +72), and F (nucleotides -63 to +72; ref. 12) with the 2C7-binding sites in a 5' position relative to the TATA sequence, were ligated with the uidA gene. The enhanced 35S promoter was ligated to coding regions of the 2C7 TF_{5ZF} protein, or the 2C7 protein fused at the C-terminal region with the herpes simplex virus transcription activation domain, VP16 or VP64. (B) Measurement of GUS activity in extracts of protoplasts 24 h after DNA transfection. The protoplasts were cotransfected with a mixture of DNAs including 5 μ g of reporter construct DNA, 20 μ g of TF_{5ZF} effector construct DNA, 5 μ g of the 35S::Luc plasmid DNA, and 10 μ g of herring sperm DNA. The results are expressed as the amount of GUS enzyme activity in picomol 4-methylumbelliferone (MU) $\text{mg}^{-1}\text{min}^{-1}$. The results are the average of three independent experiments, three samples per experiment $\pm$ SD. The results of assays that contain the effector genes are significantly different from samples containing only the correspondent reporter construct ($P < 0.05$).

the HindIII site in the polylinker of the *Agrobacterium tumefaciens* vector pGJ357. The vector pGJ357 is a modification of the plasmid pPZP200 (15). In plasmids in which the RTBV promoter was used to control expression of the effector genes, the enhanced 35S promoter was replaced by the full-length RTBV promoter (nucleotides -731 to +45) obtained from plasmid pMB9089 (12, 16).

Transient Expression Assays. Protoplasts isolated from suspension cultures of BY-2 cells (*Nicotiana tabacum* L., cv. Bright Yellow-2) were transfected via electroporation as described by Watanabe *et al.* (17). The protoplasts were cotransfected with a mixture of DNAs, including 5 μ g of reporter gene construct, 20 μ g of effector DNA construct, 5 μ g of internal control plasmid containing the chimeric gene 35S::GFP, and 10 μ g of herring sperm DNA by using a discharge of 500 μ F and 250 V through disposable 0.4-cm cuvettes. Each transient assay was repeated three times per experiment and each experiment was conducted three times.

Fig. 2. Expression of GUS activity in BY-2 protoplasts using the T fragment of the RTBV promoter and a minimal human erbB-2 promoter. (A) Diagram of the reporter and effector constructs used. T fragment (nucleotides -32 to +45) of RTBV and the minimal promoter of erbB-2 (er2; nucleotides -17 to +32) with the 2C7 recognition sites in the 5' region were fused with the uidA reporter sequence (C7T::G and C7er2::G). The effector constructs are described in Fig. 1. (B) Quantification of GUS activity from extracts 24 h after transfection. Protoplasts were cotransfected with a mixture of DNAs including 5 μ g of reporter construct C7T::G or C7er2::G, 20 μ g of TF_{5ZF} effector construct, 5 μ g of the 35S::GFP plasmid, and 10 μ g of herring sperm DNA. Results are expressed as amount of GUS enzyme activity as picomol MU $\text{mg}^{-1}\text{min}^{-1}$ compared to GFP activity. The results are the average of three independent transfections $\pm$ SD ($P < 0.05$).

Plant Transformation. The plasmids containing the effector and reporter constructs were transferred by electroporation into *A. tumefaciens* strain LBA4404. *Agrobacterium*-mediated transformation of *N. tabacum* cv. Xanthi NN was conducted as described (18). Kanamycin-resistant plants (R_0) were grown in the greenhouse and allowed to self-fertilize and R_1 seeds were collected. Plants carrying only a single insert of 35S::2C7-VP64 or C7F::G (Fig. 4A) were genetically crossed to obtain plants that contained both transgenes. Six R_0 lines were used in the crosses and R_1 seeds were collected. R_1 seeds were germinated in Murashige and Skoog medium (19) containing kanamycin 100 mg/liter, and seedlings that survived the selection were grown in soil in a greenhouse.

Analysis of Transgenic Plants. The protocol used to isolate genomic DNA from plants was a modified and simplified cetyltriethylammoniumbromide extraction method described by Futterer *et al.* (20). To assess the presence of transgenes, PCRs were used. The following oligonucleotides were used as primers: F (-18/+5), 5'-TTCGGCATTGTGAAACAAG-3'; β -glucuronidase (GUS)900-3' (907/887), 5'-ACGTAAGTCCGCATCTTCAT3-'; GUS100-5' (1693/1712), 5'-AACAAGAAAGG-GATCTTCAC-3'; C7-3' 5'-TTCAGATCAGCCGACTTAGA-

Fig. 3. Activation of GUS enzyme activity in R_0 and R_1 tobacco leaves using the F fragment of the CsVMV promoter (12). (A) Control and reporter-effector plant constructs used in the experiments. The control construct contains only the reporter gene, C7F::G or the TFs_{ZF} effector gene, 35S::2C7-VP64. In constructs that include both reporter and TFs_{ZF} effector genes in one plasmid, the effector genes are fused either to the enhanced 35S promoter or the RTBV promoter. (B) Measurement of GUS activity in leaf extracts from 30 R_0 tobacco plants containing the control or the reporter and effector constructs. Leaf extracts were taken for the assay 5 wk after the plants were potted in soil. PCR analysis was used to determine the presence of the reporter gene and the effector gene and to ensure that both genes were physically linked to each other. The results are expressed as amount of GUS enzyme activity (picomol MU mg⁻¹ min⁻¹). The results are the average of GUS activity of 30 plants $\pm$ SD. The results are significantly different to C7F::G ($P < 0.05$). (C) GUS activity in R_1 tobacco plants. Seeds were collected from 15 R_0 plants, and 20 seeds of each were germinated on Murashige and Skoog medium supplemented with kanamycin. The results of GUS activity in leaf extracts are expressed as picomol MU mg⁻¹ min⁻¹. The results presented are the average of GUS activity of the 20 plants $\pm$ SD ($P < 0.05$).

3', VP16-3', 5'-ATCTGCTCAAACTCGAAGTC-3'; VP64-3', 5'-AATTAACATATCGAGATCGAAAT-3'; BoxII RTBV, 5'-CCAGTGTGCCCTGG-3', and 35S-53 5'-tgatcaaaagtTATC-CTTCGCAAGACCT-3'. We also confirmed that in plants

Fig. 4. GUS expression in leaves of the progeny of crossed tobacco plants transformed with the reporter construct C7F::G and the activator construct 35S::2C7-VP64. (A) Diagram of the constructs used in this experiment. The construct C7F::G (Fig. 1A) and the effector were each introduced to transgenic plants. (B) Quantitation of GUS activity in leaf extracts from 5-week-old seedlings of plants that contain the single genes or that contained both genes after crossing plants described in A. The results are the average of GUS activity (picomol MU mg⁻¹ min⁻¹) of 30 plants $\pm$ SD ($P < 0.05$).

that carried both constructs on a single transgene the genes were physically linked to each other.

Fluorescence GUS Assay. Transfected protoplasts were lysed by freezing and thawing in GUS extraction buffer (pH 7.7; ref. 12) and centrifuged, and the supernatants were used for enzyme assays. GUS activity was determined by the method of Jefferson *et al.* (21). GFP activity was determined by quantifying fluorescence with 490-nm excitation wavelength and 530-nm emission wavelength (Molecular Devices). The concentration of proteins in cell extracts was determined by the dye-binding method of Bradford (22).

Histological GUS Assay. Explants sliced manually with a razor blade were stained with filter-sterilized 5-bromo-4-chloro-3-indolyl β -D-glucuronide (X-Gluc) staining solution (50 mM phosphate buffer, pH 7.0/0.5 mM potassium ferricyanide/0.5 mM potassium ferrocyanide/0.2% Triton X-100/0.5% DMSO/20% methanol/2 mM EDTA/1 mM X-Gluc) overnight at 37°C. The staining was stopped, and chlorophyll was removed by incubating in 70% ethanol. Tissues were viewed with a Nikon Eclipse 800 with bright-field optics ($\times 10$, $\times 20$ objectives).

Results

Transient Reporter Assays in BY-2 Protoplasts. *Transient expression using the CsVMV promoter.* To assess the efficacy of 2C7-based TFs_{ZF} in plant cells, we prepared three reporter and three effector gene constructs. The reporter constructs were built by using different fragments of the CsVMV promoter to drive expression of the *uidA* gene (encoding GUS): D (nucleotides -178 to +72); E (nucleotides -112 to +72); and F (nucleotides -63 to +72; ref. 12). In each case, six tandem repeats of the 18-bp DNA recognition site of the 2C7 protein was placed at the 5' end of the promoter fragment. Effector protein (TFs_{ZF}) expression plasmids used the e-35S promoter (14) to drive

expression of the 2C7 protein alone (35S::2C7), the 2C7 protein fused at its C terminus with the herpes simplex VP16 transcription activation domain (ref. 11; 35S::2C7-VP16), or the 2C7 protein fused at its C terminal with the synthetic activation domain VP64 (35S::2C7-VP64; ref. 9; Fig. 1A). VP64 is a four times repeat of the minimal activation domain from VP16.

Three independent experiments were conducted, each with three samples per experiment. BY-2 protoplasts were electroporated to introduce reporter or/and effector constructs, and GUS activity was measured 24 h later by using 4-methyl umbelliferil glucuronide as a substrate (ref. 21; Fig. 1B). In preliminary experiments, we established that GUS activity was greatest at 24 h after transfection (data not shown).

The reporter construct C7D::G in the absence of cotransfected effector (Fig. 1A) was used as to normalize the effect of various TFs_{ZF} effector genes, including constructs 35S::2C7, 35S::2C7-VP16, and 35S::2C7-VP64 (Fig. 1A). We observed less than a 2-fold increase in GUS expression in protoplasts transfected with the C7D::G plasmid and any of the effector constructs (Fig. 1B). The C7E::G reporter gene (CsVMV nucleotides −112 to +72) was more responsive to the TFs_{ZF} than was C7D::G, and significant differences in GUS expression were observed with each TFs_{ZF}. Up to 7-fold enhancement of reporter gene activity was noted with TFs_{ZF} bearing the VP16 or VP64 activation domains. Similarly the C7F::G reporter construct was also responsive to expression of the TFs_{ZF}. In this case, reporter gene activity of the TFs_{ZF} 2C7-VP64 was significantly more potent than 2C7-VP16 (Fig. 1B). Similar results were obtained when reporter and effector genes were carried on a single plasmid or on two different plasmids (data not shown).

Transient expression using the RTBV and *erbB-2* promoters. With the goal of reducing the basal level of reporter gene expression, we tested two other core promoters. The promoter from RTBV is a weaker promoter in BY-2 protoplasts as compared to the CsVMV promoter. In this case, we used a minimal RTBV promoter that contains the TATA box plus downstream sequences (T; nucleotides −32 to +45). This fragment lacks previously identified 5'cis elements (23) and was fused with the 2C7 recognition sites and the *uidA* reporter gene (C7T::G; Fig. 2A). The second construct included the TATA box region (*er2*; nucleotides −17 to +32) of the human *erbB-2* promoter with the 2C7-binding sites and the *uidA* gene (C7er2::G; Fig. 2A).

With the C7T::G reporter plasmid, we observed a 2.1-fold increase in activity with the 2C7-VP16 TFs_{ZF} and an average 4.7-fold increase with 2C7-VP64 (Fig. 2B). In studies involving the C7er2::G reporter gene plasmid, the TFs_{ZF} effector plasmid 35S::2C7-VP16 increased GUS activity an average of 4.9 times, whereas the 2C7-VP64 TFs_{ZF} increased GUS levels 27-fold (Fig. 2B). This dramatic difference in GUS activity is due in part to the very low basal level of activity of the C7er2::G plasmid.

Expression in Tobacco Plants. To test the activity of the 2C7 TFs_{ZF} system in transgenic plants, the reporter construct C7F::G was introduced to transgenic plants alone, or with the effector constructs. The effectors genes produced either VP16 or VP64 and were driven either by the e-35S promoter (for constitutive expression) or the RTBV promoter (for phloem-specific gene expression). The constructs are designated, C7F::G/35S::2C7; C7F::G/35S::2C7-VP16; C7F::G/35S::2C7-VP64; C7F::G/R::2C7; C7F::G/R::2C7-VP16, and C7F::G/R::2C7-VP64 (Fig. 3A).

After transformation using *A. tumefaciens*, kanamycin-resistant plants were grown in a greenhouse and analyzed via PCR for presence of the reporter and effector genes. Thirty transgenic plants containing the target genes were selected for each gene construct, and extracts of fully expanded leaves were used for GUS enzyme assays. As shown in Fig. 3B, when the 2C7 protein was expressed under control of the e-35S promoter, GUS activity was increased an average of 29 times compared to plants

that contained only the reporter gene. When the VP16 and VP64 activator domains were added to the 2C7 TFs_{ZF}, the amount of GUS activity increased an additional 1.5–1.9 times. When the RTBV promoter drove expression of the 2C7 gene, GUS activity increased an average of eight times, whereas the addition of the VP16 and VP64 activation domains increased GUS activity by an additional four times (Fig. 3B).

Fifteen of the 30 parental (R₀) transgenic plants lines with near average levels of GUS activity were allowed to self-fertilize, and their seeds were collected. The seeds were grown in Murashige and Skoog medium (19) supplemented with kanamycin. Twenty seedlings (R₁) were grown for 5 wk and leaf extracts were obtained, and GUS activity was determined (Fig. 3C). The average of GUS activity in plants that contained only the C7F::G transgene was set as one time. Plants that contained C7F::G/35S::2C7 had 50 times more GUS activity, and on addition of VP16 and VP64 domains to the zinc finger protein, the average GUS activity increased an additional 2–3 times, respectively. In the case of the RTBV promoter, the relative amount of GUS activity in plants with C7F::G/R::2C7 was seven times greater than in plants without the 2C7. Inclusion of the VP16 and VP64 domains increased GUS activity 8–14 times (Fig. 3C). The data collected from the R₁ generation of plants were consistent with the data from the parental R₀ lines; although the overall total amount of GUS activity was greater in the R₁ generation than in the R₀ generation of plants.

Binary transgene expression. The activity of the 2C7 TFs_{ZF} effector proteins was also tested in binary transgene expression studies. The reporter plasmid C7F::G and the TFs_{ZF} effector plasmid 35S::2C7-VP64 were introduced independently to transgenic plants. Six lines from each construct with an average GUS activity were selected for crossing and used as male and female parents (Fig. 4A). From each genetic cross, 15 seedlings were selected for further study after germination on Murashige and Skoog medium containing kanamycin and confirmation by PCR assays that they contain both plasmids. The results of GUS activity in the R₁ seedlings showed that plants that contain the TFs_{ZF} plus the reporter gene produced on average 250 times, and as much as 450 times, more GUS than plants containing only the C7F::G reporter plasmid (Fig. 4B).

Histochemical analysis. The distribution of GUS activity in plants that contained the single transgene constructs was analyzed (as in Fig. 3A). R₁ seedlings were selected on Murashige and Skoog medium supplemented with kanamycin then transferred to soil and grown in a greenhouse for 5 wk before analysis. Fresh tissue sections were obtained from the leaves, stems, and roots, and GUS activity was detected after exposing the tissues to 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), followed by the localization of indigo dye precipitates via light microscopy.

Plants containing the C7F::G reporter transgene did not contain detectable amounts of GUS activity in any tissue, as described for this promoter fragment (12) (Fig. 5a 1–4). When the plants contained the reporter plus effector transgenes C7F::G/35S::2C7, low amounts of GUS expression was detected in leaf, stem, and root. The levels of GUS expression were increased significantly by TFs_{ZF} containing the activation domains VP16 and VP64, as expected based on the results shown in Fig. 3B (Fig. 5b 1–4). By using the effector constructs that contained the RTBV promoter to drive the expression of the 2C7 gene, we did not detect GUS activity in any cells. In the presence of TFs_{ZF} containing the activation domains VP16 and VP64, GUS expression was detected in vascular tissues in leaves, stems, and roots (Fig. 5c 1–4).

Discussion

The goal of this study was to determine the effectiveness of artificial TFs_{ZF} to control gene expression in transgenic plants

Fig. 5. Histochemical localization of GUS in transgenic tobacco plants containing: the C7F::G construct (a), C7F::G/35S::2C7-VP64 (b), and C7F::G/R-2C7-VP64 (c) (constructs as in Fig. 3). (1) Cross section of leaf showing the vascular tissue of the midrib. (2) Transverse section of leaf lamina. (3) Cross section of stem tissue. (4) Whole roots. GUS activity was localized after staining of tissue slices with X-Gluc and visualizing the indigo dye precipitates formed via light microscopy. cr, cortex root; ep, external phloem; ip, internal phloem; p, phloem; ph, pith; pp, palisade parenchyma; py, parenchyma; rt, root tip; rv, root vein; sp, spongy parenchyma; and x, xylem.

and plant cells. The six-zinc finger protein system is based on specific interactions among 18 contiguous nucleotides (in this study the 2C7-binding site) and the six-zinc finger protein (the 2C7-binding protein). High theoretical specificity in sequence recognition is a key feature of these proteins and increases the potential that zinc finger proteins can be used to regulate the expression of target genes with minimal side effects. Artificial zinc finger proteins have been developed for use in animal and human cells and results of studies to date indicate a high potential for applications of this approach in gene therapy (7–11).

We first tested the efficacy of the 2C7 TFs_{ZF} system in tobacco BY-2 protoplasts as a predictor of their activity in whole plants. In this study, we used several different promoters to drive the production of the 2C7 TFs_{ZF} effector proteins and determined the role of the effectors on several reporter constructs. Reporter genes were derived from the CsVMV promoter (12), including fragment D (nucleotides –178 to +72), E (nucleotides –112 to +72), and F (nucleotides –63 to +72). In each case the 2C7 DNA-binding site was placed at the 5' end of the promoter fragment.

The C7F::G reporter gene yielded low levels of GUS activity, and cotransfection of the 2C7 TFs_{ZF} protein as a fusion with the

activation domains VP16 and VP64 resulted in approximately five times higher levels of GUS activity (Fig. 1B). C7D::G showed a lower degree of enhancement by the 2C7 TFs_{ZF} proteins, whereas C7E::G was enhanced by approximately the same proportion as C7F::G (comparing expression enhanced by 35S::2C7 vs. 35S::2C7-VP64). Differences in activation by TFs_{ZF} of the reporter genes may be caused by the differences in distance between the promoter and the 2C7-binding site. Another possible explanation is the presence of other cis elements in the longer promoters (C7D::G and C7E::G); binding of additional factors may affect the final level of gene expression (12).

We also studied the regulation of two different minimal promoters, C7T::G and C7er2::G. The C7T::G promoter, derived from the phloem-specific promoter from Rice Tungro Bacilliform Virus (nucleotides –32 to +45), has very low activity in both protoplasts and transgenic plants. In the presence of TFs_{ZF} there was a 4–5 times increase in GUS activity. The C7er2::G promoter, which is derived from the human erbB-2 promoter (9), did not produce detectable amounts of GUS activity in protoplasts. As a consequence, there were high levels of activation when 2C7 TFs_{ZF} proteins with activation domains were cotransfected with the reporter genes (5 and 27 times, respectively). These results are similar to those obtained when

the 2C7 TFs_{ZF} were used in mammalian cells with the full-length erbB-2 promoter (9).

The 2C7 TFs_{ZF} effector proteins were also active in transgenic tobacco plants. In lines that contain the 2C7 TFs_{ZF} under the control of the e-35S promoter, the addition of the activation domains increased expression of C7F::G between 1.5 and 2 times above the levels of the 2C7 protein alone (Fig. 3B). This is similar to increases in expression in BY-2 protoplasts (Fig. 1B). In the R₁ generation, the range of additive effects of the activation domains was somewhat greater, and up to a 145 times increase in gene expression was observed. It is important to note that the 2C7 TFs_{ZF}, in the absence of VP16 or VP64 domains, caused a significant increase (≈50 times) in GUS activity (Fig. 3C). These effects can be a result of changes in chromatin structure because of binding of 2C7 *per se*, that affects the binding of other factors to the DNA. It is known that local changes in chromatin structure brought about by transcription factor binding can lead to changes in gene expression (24).

When the reporter gene C7F::G and the TFs_{ZF} effector gene 35S::2C7-VP64 were introduced in separate plants, and the R₀ progeny of 12 plant lines were crossed, there was an average of ≈250 times activation of the reporter gene and a maximum enhancement of ≈450 times. This is twice the level of enhancement that was observed when the reporter and effector genes were introduced on a single plasmid.

The C7F::G reporter gene did not produce GUS activity in transgenic tobacco plants (Figs. 3 and 5; ref. 12), unless the 2C7

TFs_{ZF} proteins were coexpressed. When the constitutive e-35S promoter (21) was used to produce the TFs_{ZF} proteins, GUS activity was detected in all plant tissues. Similarly, when the phloem-specific RTBV promoter (13, 16) was used, GUS activity was restricted to the vascular system. Thus, expression of the reporter gene is controlled by the pattern of expression of the genes encoding TFs_{ZF}.

Based on our studies, we conclude that the degree of control of gene expression by synthetic zinc finger proteins will depend on the position of the binding site relative to the TATA box (or other sequences to which RNA pol II binds) and the inherent activity of the promoter that is targeted. In an accompanying paper, Guan *et al.* (25) report that a synthetic zinc finger that was designed to bind to endogenous DNA sequence 5' of the *Apetala3* gene in *Arabidopsis* exerted control of expression of this gene and changed the phenotype of the transgenic plant line. Taken together with the results reported here, we conclude that synthetic proteins can be used to manipulate the expression of foreign as well as endogenous genes and will have applications in agricultural biotechnology.

We thank Dr. Justin Stege for his advice and discussion in the early stages of this work. This study was supported by Department of Energy Grant DE-FG02-99ER20355 (to R.N.B.) and National Institutes of Health Grants CA27489 and DK61803 (to C.F.B.). M.I.O. was supported by a postdoctoral fellowship from the Ministerio de Educacion y Cultura, Spain.

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