Introduction

The ability to manipulate gene expression at will is an important tool in basic plant biology research as well as its biotechnological applications. A common approach to understanding a gene’s function involves the activation or repression of its expression and subsequent analysis of the phenotypic effects. There are several approaches for downregulation of gene expression in plants (e.g. RNAi, mutations, etc) (reviewed in Mlotshwa et al. 2002) but fewer approaches are available for upregulation. The latter usually requires the incorporation of an extra copy of the gene of interest under the regulation of either its cognate promoter or a promoter of a different expression pattern (Fang et al. 1989, Hajdukiewicz et al. 1994). In certain cases, inducible systems are used to provide regulatory flexibility with respect to developmental times and tissue types (Zuo et al. 2000). However, these approaches to upregulate the expression of a target gene do not entail the modification of the expression of its endogenous counterpart.

The Arabidopsis genome project has uncovered a number of new genes, but the individual function of the majority of them is unknown. Even when a gene displays sequence homology to a known gene in databases its functions would still require verification (Somerville and Dangl 2000). Therefore, knowledge of a gene’s function has become a demanding subject for the analysis of genomic data.

A new approach to upregulating the expression of endogenous genes involves the use of artificial zinc finger chimeras (Choo and Klug 1997, Choo and Isalan 2000, Beerli and Barbas 2002). The advantage of this technology is that artificial zinc fingers can be designed at will to bind any desired sequences in the genome, thus providing enormous potential to regulating the expression of any given gene and targeting specific DNA sequences for modification (Beerli and Barbas 2002). Usually, an artificial zinc finger contains small protein domains that are able to recognize target DNA sequences with high specificity. When linked to a transcriptional regulatory domain (e.g. activation or repression) zinc finger chimeras can be used to either upregulate or downregulate the expression of an endogenous gene and help to define the function of the encoded protein. In animal cells, artificial zinc finger chimeras

The artificial regulation of endogenous gene expression in plants is limited to only a few approaches. Here, we describe the use of artificial zinc finger chimeras to regulate the expression of a known reporter construct. The artificial zinc finger chimera TFIIIAZif is a fusion protein consisting of the four zinc fingers of TFIIIA linked through a spacer region to the three zinc fingers of Zif268. This artificial zinc finger chimera is able to bind specifically to a target DNA sequence (ZBS, zinc finger binding site) of 27 base pairs (bp). TFIIHAZif was fused to a transactivation domain from the herpes simplex virus VP16 or its tetramer VP64 to give ZF-VP16 or ZF-VP64, respectively. In transient expression assays, these two transcription activators were able to activate a target reporter gene (Luc and GFP) expressed from a minimal –46 35S promoter linked to four copies of ZBS. The activation was confirmed in transgenic plants using an inducible XVE system [Zuo et al. (2000) Plant J. 24: 265] to express ZF-VP16 or ZF-VP64. Furthermore, to test the specificity of ZF-VP64 we have compared reporter gene expression from a wild type (1xZBS) and a mutant (1xZBSmu) binding site in transgenic plants. The 1xZBS was used to express green fluorescent protein (GFP) whereas the 1xZBSmu was used to express red fluorescent protein (RFP). Upon induction of ZF-VP64 we found a much higher expression of GFP (about 33-fold) as compared to RFP expression. These results suggest that artificial zinc finger chimeras can be used to target specific DNA sequences and to regulate gene expression in plants.

Keywords: Arabidopsis — Gene expression — Regulation — Zinc finger.

Abbreviations: ACT, actin; CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; Luc, luciferase; NLS, nuclear localization signal; RFP, red fluorescent protein; VP64, tetramer of VP16; XVE, estrogen receptor-based inducible system; ZBS, zinc finger chimera binding site; ZBSmu, zinc finger chimera binding site with a mutation; ZF, zinc finger chimera.
Zinc finger chimera and gene regulation

have been successfully used to upregulate or repress the expression of the gene of interest (Zhang et al. 2000, Liu et al. 2001, Ren et al. 2002).

A zinc finger domain consists of several zinc fingers units, with each unit being able to recognize specific DNA sequences of only 3–4 base pairs (bp) (Choo and Klug 1995, Choo and Klug 1997). Critical amino acids of a zinc finger can be mutated to enable the altered protein domain to recognize any given combination of nucleotide triplet sequences. This minimal domain can be used as a basic, modular unit to assemble larger zinc finger proteins with several modules that can recognize almost any long DNA sequences. Phage display has been used to create a tagged library of zinc fingers with different triplet binding specificity (Choo and Klug 1994a, Choo and Klug 1994b, Rebar and Pabo 1994, Liu et al. 1997, Isalan et al. 1998).

To investigate the potential use of artificial zinc finger chimeras for gene regulation in plants, we have appended a transactivator domain (Beerli and Barbas 2002) to an artificial zinc finger, TFIIIAZif, (Moore et al. 2001a) and tested its ability to activate the expression of a transgene in transgenic Arabidopsis lines. In addition, we have also investigated in transgenic plants the ability of TFIIIAZif to discriminate between two target binding sites differing by only 1 bp.

Results

Zinc finger chimera and reporter constructs

We used a zinc finger chimera protein that contains four functional domains. (1) Four zinc fingers of TFIIIA (binding to 11-bp target sequence) were connected through a linker peptide to three zinc fingers of Zif268 (binding to 9-bp target sequence) and the fusion protein denoted TFIIIAZif (Pavletich and Pabo 1991, Elrod-Erickson et al. 1998, Moore et al. 2001a). This seven-finger protein is able to recognize specifically a target DNA sequence (ZBS) of 27 bp. The linker peptide between the two finger domains of TFIIIAZif is capable of spanning a 7-bp stretch of non-bound DNA (Moore et al. 2001a). (2) A short nuclear localization signal (NLS) rich in basic amino acids was included to direct the chimera to the nucleus (Choo et al. 1997). (3) A transactivation domain from the herpes simplex virus VP16 or VP64, which is a tetramer of the minimal VP16 domain, was used for gene activation (Aoyama and Chua 1997). (4) A transactivation domain from the herpes simplex virus VP16 or VP64, which is a tetramer of the minimal VP16 domain, was used for gene activation (Aoyama and Chua 1997). (4) The resulting zinc finger chimera (ZF-VP16 or ZF-VP64) was appended with a myc tag to ensure specific recognition of the expressed protein in plants by a myc antibody (Choo et al. 1997), if needed (Fig. 1A).

A detailed molecular analysis of the DNA binding specificity of the artificial zinc finger chimera has been described previously (Moore et al. 2001a). The TFIIIAZif peptide has at least a 150-fold higher DNA binding affinity than the Zif domain alone (Moore et al. 2001a, Moore et al. 2001b).

The reporter construct consists of either one copy (27 bp) or four copies (4×27 bp) of ZBS, the target DNA binding site.
of TFIIIAZif. This DNA sequence is attached to the 5′end of the CaMV 35S –46 minimal promoter, which is used to express the encoding sequence of luciferase or green fluorescent protein (GFP) (Fig. 1B). The luciferase from Photinus pyralis catalyzes the ATP/oxygen-dependent oxidation of the substrate luciferin resulting in light emission (bioluminescence) whereas GFP fluoresces under blue light (Foster and Chua 1999, Zuo et al. 2000).

**Transient expression and expression in transgenic plants**

We first used transient expression assays in onion peels to test whether ZF-VP64 and ZF-VP64 were able to activate a luc reporter construct. The β-17-estradiol-inducible XVE (Zuo et al. 2000) was used to express the zinc finger chimera and the reporter construct was cloned into a binary vector pBa002a (Moller et al. 2001). After biolistic bombardment of onion peels with DNA-coated gold particles (Bio Rad, Oxford, U.K.), the reporter construct alone was also bombarded into onion epidermal cells as a control. Bombarded onion peels were incubated with (+, panels A and B) or without (–, panels C and D) β-17-estradiol. Transient expression of luciferase was recorded after 24 expression using an imaging camera system. Similar results were obtained with three independent experiments. (A) and (C) show light images of bombarded onion peels and (B) and (D) show the corresponding luciferase images, respectively.

**Analysis of ZF-VP64 binding selectivity**

The binding specificity of zinc finger chimera is an important consideration when using multiple zinc fingers for in vivo manipulation of gene expression. We investigated this issue by comparing reporter gene expression from a wild-type and a mutant ZBS with 1 bp mutation. The wild-type ZBS was used to express GFP as a reporter gene, whereas the mutant binding site (ZBSmu) was used to express RFP (red fluorescent protein gene) as a reporter gene (Fig. 4A). The mutation in the ZBS sequence reduced the binding affinity of TFIIIAZif by 10- to 20-fold (data not shown). The zinc finger chimera construct (35S-ZF-VP64) and the reporter construct (1×ZBS-GFP or 1×ZBSmu-RFP) were transiently expressed in onion peels and after 24 h GFP or RFP fluorescence was assessed by visualization using a fluorescence microscope. We found that there was at least a 20-fold difference in the number of cells expressing GFP compared to those expressing RFP (Fig. 4B). Similar results were obtained in several independent experiments.

To investigate TFIIIAZif binding specificity in planta, Arabidopsis plants were transformed with pER8-ZF-VP64 and pBa-1×ZBS-GFP and 1×ZBSmu-RFP. Eight independent transgenic lines were obtained by selecting for hygromycin (pER8 selectable marker) and Basta (pBA002a selectable marker) double resistance, and several T3 homozygous lines were used for further studies. Transgenic lines were induced for 3 and 20 h and RNA samples were analyzed by RT-PCR. Fig. 4C shows that at 3 h induction, only GFP transcripts from the wild-type promoter were detected. However, when the expres-

![Fig. 2](https://example.com/fig2.png)
sion levels of the ZF-VP64 transactivator were increased after 20 h induction RFP transcripts from the mutant promoter were also detected. The relative level of GFP and RFP expression was about 33-fold reflecting the difference in the binding affinity between ZBS and ZBSmu for ZF-VP64, which is about 10- to 20-fold. ACT2 transcript levels were not affected by the inducer and were used as internal controls in these expression experiments. Analysis of several independent transgenic lines produced similar results. Our results indicate that ZF-VP64 is able to target a wild-type DNA binding site with a greater affinity than a mutant DNA-binding site with a reduced affinity.

Discussion

Previous work with animal cells has demonstrated the use of zinc finger chimeras to regulate expression of reporter gene constructs in transient transfection assays. In only a few cases, have zinc finger chimeras been shown to regulate the expression of endogenous genes in stably transformed mammalian cells, like the human erb-2 (Beerli et al. 2000). Furthermore, recent work has demonstrated the activation of the human erythropoietin gene and the gene encoding the vascular endothelial growth factor A in stable transformed human embryonic cell lines (Zhang et al. 2000, Liu et al. 2001). In addition, selective repression of a splice variant (PPARγ2) of the nuclear hormone receptor in mouse cell lines has been achieved using similar technology (Ren et al. 2002).

As a first step to using zinc finger chimeras to regulate endogenous plant gene expression we have investigated the ability of ZF-VP16 and ZF-VP64 to activate reporter gene constructs containing at their promoter region one or four copies of ZBS by transient assays, and more importantly, in transgenic Arabidopsis plants. We show here that the two zinc finger chimeras can be expressed in plant cells and activate a reporter transgene containing its cognate binding site, ZBS. Transgenic plants expressing these zinc finger chimeras were morphologically normal throughout development and did not display any adverse physiological effects when their expression was induced. These results confirm previous studies in animal systems (Kim et al. 1997, Kim and Pabo 1998, Choo et al. 1997, Beerli et al. 2000, Urnov and Rebar 2002) and extend the utility of zinc finger chimeras to plant systems.

To determine the specificity of interaction between artificial zinc finger chimeras and target DNA binding sites, we
investigated the relative expression levels of two reporter transgenes, one containing a wild-type 27-bp ZBS whereas the other contained a mutant ZBS with a 1-bp mutation. Previous in vitro binding assays have demonstrated that the binding affinity of the wild-type ZBS is about 10- to 20-fold higher than that of the ZBSmu. We varied the expression of the ZF-VP64 transactivator by using different inducer concentration. At low ZF-VP64 expression levels, reporter transgene expression was detected only from the wild-type ZBS. However, when the expression level of ZF-VP64 was increased, expression from the mutant binding site, ZBSmu, was also observed. The relative level of expression from the ZBS-linked and ZBSmu-linked promoters reflects the difference in their binding affinities for the transactivator.

After the completion of this work, two very recently published papers reported on the use of artificial zinc fingers to regulate gene expression in transgenic plants (Guan et al. 2002, Ordiz et al. 2002). Collectively, our results and the results of their work indicate the feasibility of modulating endogenous plant gene expression using artificial zinc finger chimeras for basic plant biology research, as well as for biotechnological applications.

Our experimental designs differ in several aspects from those reported in the two papers (Guan et al. 2002, Ordiz et al. 2002). First, the zinc finger protein used in our experiments recognizes a target of 27 bp and consists of a seven-finger protein with a linker peptide spanning 7 bp separating the two zinc finger binding regions (Fig. 1). This permits a longer DNA sequence to be recognized in the genome and thus potentially reducing non-specific binding. Moreover, the use of the linker permits more flexibility in the zinc finger design and increases its specificity. By contrast, the two recent reports used a zinc finger protein that recognizes a 18-bp zinc finger binding site (Guan et al. 2002, Ordiz et al. 2002). Second, in our transgenic experiments we used the XVE inducible system (Zuo et al. 2000) that enabled us to regulate the expression levels of the artificial zinc finger chimera by controlling the inducer concentration. In addition, we can induce the artificial zinc finger at any stage of development or growth of the plants, avoiding

![Fig. 4](https://academic.oup.com/pcp/article-abstract/43/12/1465/1915004/1915004)

Specificity of gene activation of ZF-VP64. (A) The first reporter construct contains one copy of ZBS; the −46 35S promoter (−46 to +9) and the GFP-coding sequence (upper). The second reporter construct with the mutant binding site (ZBSmu) is similar to the first except that the DNA binding site contains a single base mutation (C by G) and RFP-coding sequence as a reporter gene. For more details see Materials and Methods. (B) Transient expression of pE88-ZF-VP64 and reporter constructs 1xZBS-GFP and 1xZBS-mu-RFP (Fig. 3A) were introduced into onion peels by biolistic bombardment (Bio Rad, Oxford, U.K.). After 24 h of treatment with β-17-estradiol (30 μM) transient expression of GFP or RFP was analyzed by a fluorescence ZEISS microscope system. 1xZBS-GFP contains the wild-type DNA binding site (ZBS) whereas 1xZBS-mu-RFP contains the mutated DNA binding site (ZBSmu) with 1-bp mutation. (C) Specificity of the zinc finger chimera transcription factor in transgenic plants. Transgenic plants carrying the transgene XVE-ZF-VP64, 1xZBS-GFP and 1xZBS-mu-RFP were exposed to β-17-estradiol (30 μM) for 0, 3 and 20 h. RNAs (200–500 ng) were extracted from the treated plants and used for each of the indicated RT-PCR reactions. Three independent experiments were performed and results from a representative experiment were shown.
problems reported by Guan et al. (2002), in which they were not able to express constitutively the ZFP\textsuperscript{VP64} plasmid and had to use a flower-tissue specific promoter instead. Third, we evaluated the implications of a single nucleotide mismatch in the zinc finger binding site and demonstrated the specificity of the zinc finger binding in transgenic plants. This specificity issue was not addressed by the two recent papers.

**Material and Methods**

**Reagents**

DNA sequences encoding sequence zinc finger chimeras containing the DNA binding domain pTFIIIAZif with an NLS sequence, the VP16 and VP64 activator region, and ZBS, the target DNA binding site of TFIIIAZif were prepared as previously described (Moore et al. 2001a). cDNA encoding pTFIIIAZif-VP16-myc and pTFIIIAZif-VP64-myc were referred as ZF-VP16 and ZF-VP64, respectively.

**Expression vectors for zinc finger chimera**

The zinc finger chimeras were expressed using a 17-β-estradiol inducible system, pER8 (Zuo et al. 2000). pER8 plasmid DNA was digested with Mlu\textsuperscript{I} and Spe\textsuperscript{I} (New England BioLabs, Beverly, MA, U.S.A.). A clone for each construct was verified by restriction analysis. All constructs were introduced into Agrobacterium tumefaciens (New England BioLabs, Beverly, MA, U.S.A.). DNA sequences encoding zinc finger chimeras containing the CaMV 35S promoter –46 to +9 upstream of luc (New England Biolabs, Beverly, MA, U.S.A.) and 20 mg ml\textsuperscript{-1} sodium hypochlorite/0.01% Tween-20 (Sigma, St. Louis, MO, U.S.A.) were used for plant transformation. Plants were surface-sterilized by treating them with a solution of 1.5% sodium hypochlorite/0.01% Tween-20 (Sigma, St. Louis, MO, U.S.A.) and grown hydroponically for 2 d (Sanchez and Chua 1997). Similar procedures were used to clone the ZF-VP16 and ZF-VP64 into the AscI and SpeI sites of pER8.

**Reporter construct for the zinc finger chimera**

The pKL+1 plasmid was used for the construction of reporter plasmids (Foster and Chua 1999). The plasmid contains a minimal promoter –46 to +9) upstream of luc (New England Biolabs, Beverly, MA, U.S.A.) and 20 mg ml\textsuperscript{-1} sodium hypochlorite/0.01% Tween-20 (Sigma, St. Louis, MO, U.S.A.). A clone for each construct was verified by restriction analysis. All constructs were introduced into Agrobacterium tumefaciens strain ABI (Aoyama and Chua 1997). Similar procedures were used to clone the ZF-VP16 and ZF-VP64 into the AscI and SpeI sites of pER8.

**Plant transformation**

Plants of Arabidopsis thaliana ecotype Landsberg erecta were transformed with Agrobacterium using the vacuum infiltration procedure (Bent et al. 1994). Seeds collected from the vacuum infiltrated plants were surface-sterilized by treating them with a solution of 1.5% sodium hypochlorite/0.01% Tween-20 (Sigma, St. Louis, MO, U.S.A.) for 10 min and washed three times with sterile water. Sterilized seeds were then resuspended in 0.1% agarose and sown on Petri dishes containing A medium (full-strength Murashige and Skoog salts, pH 5.7, 1% sucrose, solidified with 0.8% Bactoagar, Gibco BRL, Grand Island, NY, U.S.A.) and 20 μg ml\textsuperscript{-1} hygromycin B (Sigma, St. Louis, MO, U.S.A.). After vernalization for 4 d in the dark, the plated seeds were transferred to a growth chamber maintained at 22°C under 16 h light/8 h dark. Transgenic T1 seedlings were selected on plates containing hygromycin (20 mg ml\textsuperscript{-1}) for pER8 and Basta (10 μg ml\textsuperscript{-1}) for pBA002a. After 2–3 weeks of growth, the presence of transgenes in putative transgenic plants was confirmed by PCR analysis.

**β-17-estradiol treatment**

β-17-estradiol (Sigma, St. Louis, MO, U.S.A.) was dissolved in dimethylsulfoxide (DMSO) to make a 100 mM stock solution and stored at –20°C. To monitor transgene expression, putative transgenic plants were surface sterilized and sown on Petri dishes as described above. After vernalization at 4°C for 4 d the plates were incubated for 2 weeks in a growth chamber maintained at 22°C 16 h light/8 h dark. Seedlings were transferred from the plates to liquid media (full-strength Murashige and Skoog salts, pH 5.7, 1% sucrose, Gibco BRL, Grand Island, NY, U.S.A.) and grown hydroponically for 2 d (Sanchez and Chua 2001). Fresh media containing either DMSO or β-17-estradiol (30 μM) was added and treated plants were removed at the designated time points, washed and then frozen in liquid nitrogen. Parallel experiments were carried out with the vector control transgenic lines under similar conditions.
RNA analysis

Total RNA was isolated from seedlings and adult plants using a Qiagen RNA purification kit (Qiagen, Valencia, CA, U.S.A.). RNA gel blot analysis was carried out according to Sanchez and Chua (2001). Each lane contained 10 µg of total RNA. DNA fragments encoding TFIIIAZif, luciferase and 18S rDNA were obtained by PCR amplification with Pfu polymerase as described above. Fragments were purified using the Qiagen Gel extraction protocol (Qiagen, Valencia, CA, U.S.A.) and labeled with [32P]dCTP and [32P]dATP by random priming (Amersham, Arlington Heights, IL, U.S.A.). Hybridization signals were quantified using the Phosphoimager STORM system (Molecular Dynamics) and the data analyzed with the Image Quant v1.1 program.

Luciferase imaging and light microscope

Microscopic analysis of GFP and RFP fluorescence was done using an Axioskop (Zeiss, Germany) according to Kost et al. (1998). Onion peels and 3-week-old seedlings were sprayed with 2.5 mM luciferin (Promega, Madison, WI, U.S.A.) containing 0.05% Triton X-100 and the luciferase activity monitored by photon counting. Video images (5 min) were captured in the gravity mode using a intensified CCD camera and coupled photon-counting MethaMorph software (Universal Imaging Corporation, PA, U.S.A.)

RT-PCR

RT-PCR was performed using the Titan one tube RT-PCR system (Roche Molecular Biochemicals, Mannheim, Germany) according to protocols described by the manufacturer. For PCR amplification, 20–25 cycles were used for each of the samples except the control actin-2, which was detected at 19 cycles. The primers used for PCR amplification of TFIIIAZif were 5'-CCAAGCTTACGGGAGAAGG CGGTCCGCGTTG G-3' and 5'-CCACGACTCTTACACAGATCGC GGCA-3'; the luciferase gene was amplified with primers 5'-CCGGATT CTACCTGGACAA GCTGCTGACGCGGAG-3'; primers used for RFP were 5'-CCCTGCAAGCCCG GACGCTGCAAGACGCGG-3' and 5'-CA GTTGGAAATCTAGCTGCGGCGCTAC-3'; and for actin-2 (U37281) were 5'-GCCGGAACCCTTTG GATGTAAGCCGCTC-3' and 5'-ACAGTATTGACATGGTACATGGGCGC-3'. Amplified DNAs were separated on 1% agarose gels. All DNA bands resulting from the RT-PCR reaction were tested for RNAase A sensitivity. 32P-labeled primers were used to quantify the amplified genes using the radioactive methods described by Pfaffl et al. (1998).

Acknowledgments

We thank Dr. Peter Hare and Dr. Guofo Li for critical reading of the manuscript.

References


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(Received October 9, 2002; Accepted November 8, 2002)