Controlling gene expression in plants using synthetic zinc finger transcription factors

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Summary

Synthetic zinc finger proteins can be fused to transcriptional regulatory domains to create artificial transcription factors that modulate the expression of a specific target gene. Recent studies have demonstrated that synthetic zinc finger domains can be constructed to bind DNA sequences with a high degree of specificity. To devise a general strategy for controlling plant gene expression with artificial transcription factors, a rapid transient assay was developed to test the regulatory activity of synthetic zinc finger transcription factors (effectors) on target plasmids (reporters) in plant cells. Effective activation was demonstrated with zinc finger proteins fused to a derivative of the VP16 activation domain. The mSin3 interaction domain (SID) of the human MAD1 protein provided moderate repression of target reporters. Unlike many naturally occurring transcription factors, these synthetic effectors exhibit a strong dependence on binding site position. Reporter genes that are stably integrated into plant cells responded similarly to transiently transfected reporter plasmids, verifying that this assay accurately reflects the behavior of these transcription factors on an endogenous target within the context of chromosomal DNA. These results provide evidence that synthetic zinc finger proteins can be used to manipulate the expression of endogenous genes in plants.

Keywords: artificial transcription factors, gene regulation, transcriptional activation, transcriptional repression, zinc finger protein, plant gene expression.

Introduction

Studies of transcription factors have shown that they are typically designed in modular fashion with a DNA binding domain and a transcriptional regulatory domain (Ayer et al., 1996; Margolin et al., 1994; Triezenberg et al., 1988). These experiments also demonstrated that the regulatory domains can function when fused to different DNA binding domains to generate chimeric transcription factors with altered gene specificity. In a typical transcription factor protein, the DNA binding domain directs the activity of the regulatory domain to the specific genetic locus to alter the expression of the target gene. Thus, it is possible to generate a synthetic transcription factor by fusing a genespecific DNA binding domain to a transcriptional regulatory domain. Recent advances in synthetic zinc finger protein (ZFP) research have led to the design of target-specific DNA binding proteins and construction of transcription factors with novel specificity (Beerli and Barbas, 2002). Once developed, this technology can be used to design artificial transcription factors to control the expression of any plant gene. The application of this technology ranges from purely scientific studies to the development of valuable agronomic traits in crop plants.

Zinc finger proteins have been shown to bind specific DNA sequences with high affinity and act as the DNA binding domain of transcription factors (Cook et al., 1999; Margolin et al., 1994). The Cys2His2 family of zinc finger
DNA binding domains has been shown to be particularly amenable to rational manipulation to modify binding specificity and several methods have been developed to construct zinc finger DNA binding domains specific for a given target sequence (Choo and Klug, 1994; Desjardais and Berg, 1992; Dreier et al., 2001; Liu et al., 1997; Rebar and Pabo, 1994; Wu et al., 1995). Each finger motif is about 30 amino acids (AA) long and contains cysteine and histidine residues that coordinate a zinc ion to stabilize the folding of this simple βα domain. Using current approaches, it is possible to construct 6-finger (polydactyl) proteins that specifically bind targeted 18-bp DNA sequences (Beerli et al., 2000; Beerli et al., 1998; Dreier et al., 2001; Liu et al., 1997; Ren et al., 2002).

Studies of a ZFP constructed to bind to an 18-bp target within the human erbB-2 promoter (Beerli et al., 1998) have explored the efficacy of a variety of activator and repressor domains in mammalian cells. These studies led to the development of synthetic transcription factors that significantly increased or decreased the expression of the endogenous human erbB-2 and erbB-3 genes (Beerli et al., 2000). Several other artificial transcriptional factors, constructed by similar methods, have also been shown to regulate endogenous genes (Dreier et al., 2001; Liu et al., 2001; Ren et al., 2002; Zhang et al., 2000), demonstrating the potential application of transcription factors customized for a specific gene. However, before this technology can be exploited as a robust tool for controlling gene expression in plants, a number of parameters need to be examined. Some of the factors that could influence the efficacy of a transcription factor include: DNA binding site location within the target promoter; choice of regulatory domain; specificity of regulation; and non-target effects.

To date, the studies of regulation of gene expression by synthetic ZFP transcription factor proteins have been performed exclusively in mammalian cells. The DNA binding activity of the ZFP is expected to work in plants, but interactions with endogenous transcriptional machinery may be different than in mammalian cells. Because most research on generating chimeric transcription factors has been done in mammalian and yeast systems, it will be necessary to identify plant-specific regulatory regions that can function when fused to zinc finger DNA binding domains. The herpes simplex virus VP16 domain (Sadowski et al., 1988) and its derivatives have been shown to function in plants and other systems to activate expression when fused to the Gal4 DNA binding domain (Estruch et al., 1994). It is likely that the VP16 domain will function as an effective transcriptional activation domain when fused to ZFPs and expressed in plants.

The identification and characterization of repressors of transcription has proven to be a more difficult task. To date, no plant repressors have been identified that behave in a modular fashion. However, it is possible that repression domains already identified in other systems may function well in plants. The mSin3 interaction domain (SID) (Ayer et al., 1996) and the KRAB domain (KRAB) (Margolin et al., 1994) have been fused to ZFPs to generate effective transcription factors for use in mammalian cells (Beerli et al., 2000; Beerli et al., 1998). The SID domain acts by recruiting a histone deacetylase complex that ultimately modifies local chromatin structure (Eilers et al., 1999). The KRAB domain acts by binding the KAP-1 co-repressor, which in turn serves as a scaffold to recruit proteins for histone deacetylation and methylation (Schultz et al., 2002). These activities recruited by the KRAB domain are thought to modify the chromatin structure in such a way as to repress transcription.

The goal of this study was to demonstrate the use of synthetic ZFPs in artificial transcription factors to control gene expression in plants. Furthermore, experiments were performed to test the specificity of regulation and explore the effects of binding site location, promoter strength and different regulatory domains on gene regulation by chimeric transcription factors. A rapid assay was developed to test the effects of zinc finger transcription factors (effectors) on the expression of a target gene (reporter) in tobacco and maize protoplasts by transient expression of an effector protein and a reporter plasmid. In tobacco, the effector protein was expressed using a TMV-based vector that provided extremely high transfection efficiency and protein expression. Various effector constructs were made with the well-studied C7 ZFP (Liu et al., 1997; Wu et al., 1995). The C7 zinc finger contains three zinc finger domains and binds nine contiguous bases. Linking two C7 proteins together generated a 6-finger 2C7 protein, which has been shown to bind specifically and tightly to 18 contiguous nucleotides (Liu et al., 1997). The 2C7 zinc finger was used to construct different effector proteins and tested against a variety of reporter constructs to investigate the behavior of these novel zinc finger transcription factors and to develop a strategy for regulating targeted genes in plants.

**Results**

**Transient activation of luciferase reporter in plant cells**

Activation by chimeric transcription factors that contain the VP16 activation domain has been previously demonstrated in plants (Moore et al., 1998). In the present study, effector proteins were constructed by fusing the 3-finger C7 ZFP or the 6-finger 2C7 protein to four tandem repeats of the minimal VP16 activation domain (Beerli et al., 1998) (Figure 1b). These effectors were expressed using a transient expression vector derived from TMV. Effectors were tested against a luciferase (LUC) gene reporter that contains a minimal promoter with six repeats of the 2C7 binding site.
(Figure 1a). Tobacco BY-2 protoplasts were co-transfected with viral RNA encoding the effector proteins and reporter plasmid DNA. To measure the activity of the effector proteins on reporter expression, the LUC activity is normalized to Renilla luciferase (RL) activity that was elicited by the control plasmid (pCAT-RL, Figure 1a). The relative activity is calculated by dividing the normalized LUC activity obtained with effector expression by the normalized LUC activity without any effector expression.

The 2C7-VP64 (a 6-finger ZFP) activates this minimal promoter over 20-fold relative to the unactivated level, while the 3-finger C7-VP64 effector provides over 80-fold activation (Figure 2a). There are two possible explanations why the 3-finger construct promotes higher levels of activation. First, the reporter construct contains 12 possible binding places for the 3-finger ZFP C7-VP64 and only six possible binding places for the 6-finger ZFP 2C7. Thus, C7-VP64 may bring a greater number of activation domains to the target and activate transcription more effectively. Alternatively, the 3-finger ZFP C7-VP64 may accumulate to higher levels than the 6-finger ZFP 2C7. It has been observed that 3-finger ZFPs tend to accumulate to higher levels of protein than 6-finger ZFP when expressed in mammalian cells (C. Barbas, unpublished data).

We also performed similar activation experiments in maize cells. In this system, the 2C7-VP64 activator was expressed with the ubiquitin promoter (Cornejo et al., 1993) on a plasmid construct. The 2C7-VP64 activator...
was co-transformed with two different reporters with minimal promoters containing the C7 binding sites (Figure 1a). The 2C7-VP64 protein activated the p50C7 reporter 146-fold and the pC7rbTATA reporter 40-fold (Figure 2b). This indicates that ZFP transcription factors perform well in monocot as well as dicot cells.

**Position dependence of activation**

To determine if the location of the zinc finger binding site within the target promoter affects the function of these effector proteins, a series of luciferase reporters were constructed using the constitutive Cassava Vein Mosaic Virus (CsVMV) promoter (Verdaguer et al., 1996) with 6 × 2C7 binding sites located at different positions relative to the transcriptional start site (TATA box) (Figure 1a). The activation of these reporters by the C7-VP64 and 2C7-VP64 activators was tested in tobacco BY-2 protoplasts (Figure 3). As the C7 binding site is moved to a greater distance from the TATA box, activation is significantly reduced. In the p50C7 reporter, the 6 × 2C7 binding sites are 443 bp away from the start of transcription; however, there is very little activation with this construct. In the p5C7 reporter, the binding sites are only 63 bp away from the TATA box and luciferase expression is activated about fivefold by the 2C7-VP64 protein. When the binding site is positioned past the start of transcription at the very 3′ end of the promoter (p3′C7), the 2C7 effector binding seemed to inhibit expression, even when fused to the VP64 activator. The binding of multiple effector molecules in this location may interfere with the

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**Figure 2.** Activation of a reporter gene in tobacco BY2 cells with 3- or 6-finger ZFP. (a) Activation of prbTATA and pC7rbTATA reporters (Figure 1a) following co-transformation with C7-VP64 (black bar) or with 2C7-VP64 (white bar) activators (Figure 1b). (b) Activation of p5′C7F and pC7rbTATA reporter plasmids (Figure 1b) in maize protoplasts with 2C7-VP64 activator protein.

**Figure 3.** Position dependence of reporter activation on binding site location. Activation of luciferase following co-transformation of each of the seven CsVMV reporters (Figure 1a) with 3-finger activator C7-VP64 (black bar) or with 6-finger activator 2C7-VP64 (white bar). Fold activation relative to the activity observed with no effector protein is listed under each column.

activity of the RNA polymerase complex and subsequently reduce the expression of the reporter gene.

The inherent strength of the target promoter may affect its regulation by exogenous transcription factors. p5’C7F and pC7ΔE have the zinc finger binding site in the same location relative to the TATA box in the promoter (Figure 1a), and pC7ΔE has an additional fragment of the CsVMV promoter that is missing from the p5’C7F construct. This results in much higher basal luciferase activity with the pC7ΔE reporter than with the p5’C7F reporter (data not shown). When activated by C7-VP64 or 2C7-VP64 effectors, the stronger pC7ΔE reporter is activated to a lesser degree than the weaker p5’C7F reporter (Figure 3). Furthermore, when a minimal promoter (pC7rbTATA) with a very low basal activity is used, very high level of activation was observed (Figure 2a). The basal levels of p5’C7F and pC7ΔE luciferase activity (LUC/protein) exhibited more than 100-fold difference (data not shown), but the activated levels by these reporters varied by less than fivefold. This may indicate that there is a maximal level of expression for this promoter/activator system.

Repression of the luciferase reporter

Unlike other systems, regulatory domains that repress transcription in plants when fused to different DNA binding domains have not been identified. However, the application of foreign transcriptional activator domains in planta indicates that repression domains from other systems may function in plants. We tested two repressor domains isolated from mammalian transcriptional regulators: SID (Ayer et al., 1996) and KRAB (Margolin et al., 1994). Both domains were fused to the N-terminus of the 2C7 ZFP (Figure 1b). As a control for non-specific repression, a non-binding ZFP (e2c) was fused to the SID domain. The repressor and reporter constructs were then co-transformed into tobacco BY2 cells to determine their effects on transcription. The 2C7-SID exhibited fivefold repression of the p5’C7F and pC7rbTATA reporters (Figure 4). This level of repression is approximately as effective in plant cells as in mammalian cells (Beerli et al., 1998). The 2C7-KRAB effector shows no specific repression. These results are contrary to the situation in mammalian cells where the KRAB domain proved to be a more effective repressor than the SID domain (Beerli et al., 1998). In our experiments, some degree of non-specific repression was observed with the SID repressor. The e2c-SID protein caused moderate levels of repression with the p5’C7F and pC7rbTATA reporters, even though there are no specific binding sites for the e2c protein in these constructs (Figure 4).

As with activation, it appears that stronger promoters may be more difficult to repress than weak promoters. The strong pC7ΔE reporter is repressed at a lower level than the weaker p5’C7F reporter (Figure 4).

**Figure 4.** Repression of reporters with 6-finger ZFPs. The e2c-SID, 2C7-SID, and 2C7-KRAB effector proteins (Figure 1b) were expressed in tobacco BY-2 protoplasts in combination with three reporter plasmids, pC7ΔE, pC7rbTATA, and p5’C7F (Figure 1a). The levels of repression to reporter pC7ΔE, p5’C7F, and pC7rbTATA were represented as white, striped, and black bar, respectively. Fold activation relative to the activity observed with no effector protein is listed under each column.

**Specificity of regulation**

In developing a system to regulate gene expression, it is important that the desired effects are specific to the target gene. The specificity of each of these effector constructs was investigated using an internal control plasmid. In these experiments, a control plasmid expressing the Renilla luciferase (pCAT-RL) was co-transfected with the effector and reporter plasmids. Because the pCAT-RL control plasmid has no 2C7 zinc finger binding sites, the RL activity should be unaffected by the expression of 2C7 transcription factors. Thus, the pCAT-RL was co-transfected along with the target reporter plasmid to serve as an internal control for transformation efficiency. However, if there is non-specific activity of these effectors, the RL activity may be affected. Instead of normalizing luciferase activity to RL activity, the LUC and RL activities were normalized to protein levels in the cell extracts. These protein-normalized activities were then divided by the activities obtained in the no effector condition to generate a measure of relative activity. A perfectly specific activator should have a relative RL/protein of 1.0 and the relative LUC/Protein and LUC/RL should be the same. For an activator with non-specific activity, LUC/protein normalization should reflect the total level of activation (non-specific and specific), RL/protein should reflect non-specific activation, and LUC/RL should measure only specific activation.

When expressed with the p5’C7F reporter, all of the effectors tested in this study had some level of non-specific
activity (Table 1). Even the expression of C7-GFP (C7 ZFP fused to GFP) appeared to affect a non-specific activation of 38%. It is suggested that binding of the ZFP upstream of this promoter causes modification of DNA structure, allowing more efficient initiation of transcription. For some of the effector constructs, the e2c (Beerli et al., 1998) zinc finger was used as a control. The e2c zinc finger binds specifically to the erbB-2 promoter and should not bind in these reporter plasmids. In our experiments, the e2c-SID effector caused modest repression (17%) of the target LUC activity (Table 1). However, the 2C7-SID effector represses the luciferase target reporter but not the RL control reporter fivefold, indicating that it is a specific repressor. The 2C7-KRAB effector causes significant (twofold) repression of both the luciferase target reporter and the RL control reporter indicating that it is non-specific. In Figure 3, repression by the 2C7-KRAB effector protein was calculated by normalizing LUC to RL activity. From these calculations, one would conclude that there was no repression by the 2C7-KRAB. However, analyzing both the LUC and RL activities separately indicates that there is a non-specific repression of both reporters. These calculations illustrate the need to accurately characterize the specificity of transcription factors for non-target effects.

Effector constructs designed to activate transcription used the VP64 activation domain. The non-target e2c-VP64 effector exhibits some non-specific activation of the target reporter and the 2C7-VP64 effector activates the RL expression to a low degree (Table 1). However, the strong activation of p5’C7F luciferase activity by both the 2C7-VP64 and the C7-VP64 effector protein indicates that this is a relatively specific activator.

Regulating expression of integrated versus non-integrated genes

One concern of using a transient assay to test synthetic ZFP-based artificial transcription factor is that the results may not be applicable to endogenous gene targets. It can be argued that the chromatin structure of a transiently transfected plasmid will be different from that of an endogenous sequence located on a chromosome. Thus, the binding and regulation by these effector proteins may behave differently on endogenous targets. This is especially true for some repression domains that are thought to function by altering the structure of the chromatin around their target gene. To address this issue, transgenic tobacco BY-2 cell lines were established with the pC7AE target reporter genes stably integrated into the plant genome. Transient expression assays were performed with protoplasts derived from these cell lines to test the activation and repression of an integrated target by synthetic ZFP-based artificial transcription factors expressed from the TMV vector. The integrated reporter appears to respond to the effector constructs in essentially the same manner as when the cells are co-transfected with the plasmid reporter (Figure 5). Thus, a transiently transfected plasmid target behaves similarly to an integrated target. This finding suggested that this rapid co-transformation assay can be reliably used to characterize new effector designs and putative regulatory domains.

Discussion

The experiments described in this paper demonstrate that artificial transcriptional factors constructed with synthetic ZFPs can activate and repress expression of a target gene. Combined with the ability to create ZFP domains to bind any DNA sequence, it may be possible to manipulate the expression of endogenous plant genes with this approach. However, there are many issues to be resolved before this technology can be used as a general method to alter the expression of any given target gene in plants. The selection of DNA binding domains and transcriptional regulatory domains will influence the efficacy, specificity, and reliability of the artificial ZFP transcription factors. Thus, it is important to evaluate the behavior of different effector constructs in studies to regulate endogenous genes.

In the many published studies of chimeric transcription factors, very little has been done to address the issue of non-specific regulation of non-target genes (Ayer et al., 1996; Estruch et al., 1994; Hsieh et al., 1999; Jones et al., 1998; Margolin et al., 1994; Nan et al., 1998). The specificity of any effector protein that is used to regulate gene expression is of significant concern if they are to be used in gene therapy or in agriculture. Therefore, it will be very important to identify regulatory proteins and effector designs that provide effective regulation of the target gene without significantly affecting expression of non-target genes.

Table 1 Specificity of zinc finger transcription factors

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<tr>
<th>Controls</th>
<th>Repression</th>
<th>Activation</th>
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<tr>
<td>No effector</td>
<td>GFP control</td>
<td>C7-GFP</td>
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<tr>
<td>LUC/Protein</td>
<td>1.00</td>
<td>1.38</td>
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<tr>
<td>RL/Protein</td>
<td>1.00</td>
<td>0.98</td>
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<tr>
<td>LUC/RL</td>
<td>1.00</td>
<td>1.44</td>
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The specificity of DNA binding for synthetic ZFP has been shown to be very high in vitro (Segal et al., 1999). It remains to be seen how in vivo DNA binding specificity correlates with in vitro regulatory behavior. A 6-finger polydactyl ZFP binds a contiguous 18-bp DNA sequence and if the ZFP is specific for those 18 bp, the chances of another target site existing elsewhere in the genome is low, even in the large genomes of humans and higher plants. In our studies, a 3-finger ZFP effector protein provided more activation than a similar 6-finger protein. However, a 3-finger ZFP will bind a 9-bp DNA target, a factor that is expected to increase the likelihood of binding to multiple genomic locations, and increases the likelihood of non-specific effects of the ZFP.

Clearly, the specificity of binding of the ZFP to the target DNA will be a major determinant of the specificity of the transcriptional regulation. However, it is possible that the choice of regulatory domains could also affect specificity. In these experiments, almost all of the effector proteins, including proteins that lack regulatory domains, exhibited some level of activity. It is possible that the level of effector protein expression could influence the degree of regulation. All the experiments in this study used the TMV viral vector for expression of the effector proteins that can lead to high levels of protein production in plant cells (Padgett et al., 1996). Lowering the level of effector expression may result in more specific regulation. Therefore, it may be important to tightly control effector expression to achieve optimum regulation with minimal non-target effects.

The optimal location of the site for ZFP binding within a target gene is not well characterized. While naturally occurring transcription factors can act over long or short distances (Gallie, 1998; Stein et al., 2000), the experiments described here indicate that ZFP-based artificial transcription factors are highly dependent on the distance of the binding site from the start of transcription. Maximum regulation of a strong viral promoter (CsVMV) was obtained when the binding site was placed close to the start of transcription as possible. However, when the binding site was placed downstream of the transcriptional start site, regulation was not effective.

It is possible that modification of transcription factor design could affect position dependence. For example, inserting a long flexible linker of amino acids between the DNA binding domain and the regulatory domain may allow the regulatory domain more freedom to influence transcription from a greater distance. Also, other activation domains may function differently and have better activity from a distance. Position dependence may seriously limit the possibilities when designing new ZFP-based artificial transcriptional factors. On a positive note, this may imply that zinc finger effectors will not affect transcription of endogenous genes that are adjacent to the target gene.

The effect of promoter strength on regulatory activity was examined in this study by using a number of derivatives of the CsVMV promoter. These results indicate that stronger promoters are harder to regulate than weaker ones. To demonstrate that the ZFP technology can be used as a general method for modifying gene expression in plants, the promoter dependence of effector proteins needs to be evaluated. We are currently targeting several different promoters since every gene is likely to have a unique set of control elements that respond differently to certain regulatory domains. If the regulatory domains currently being used exhibit strong promoter dependence, it may be necessary to search for other domains with more general regulatory activity.
When fused to the 2C7 zinc finger, the VP64 regulatory domain provides effective, specific activation of target genes in both monocot and dicot plant cells. This is not too surprising since VP16 activation domains have been successfully demonstrated to function in plant systems (Moore et al., 1998). Thus, the VP64 is a suitable choice for constructing artificial transcriptional activators based on synthetic ZFPs. However, there may be other plant-specific activation domains that work as well or better than VP64.

Identifying effective repression domains for use in constructing artificial transcriptional repressors based on synthetic ZFP is a greater challenge. To date, no plant proteins have been identified that contain a domain that can serve as a modular transcriptional repressor when fused to a DNA binding domain. Two mammalian repression domains, SID and KRAB, showed only limited function in plants. The KRAB domain produced moderate, non-specific repression in plants cells, while the SID domain provided moderate, but specific repression in plant cells. It is possible that lower levels of KRAB effector protein expression could increase the specific repression. Homology searches reveal no close KAP-1 homologs in public plant sequence databases (data not shown). Because the KRAB domain mediates repression by binding the KAP-1 co-repressor in mammalian cells, the lack of a similar protein will prevent KRAB from functioning in plant cells. Recent studies have identified a number of plant proteins capable of repressing transcription in plants (Jin et al., 2000; Raventos et al., 1998). The transient assay developed here could be used to quickly identify and characterize any modular repression domains in these proteins that can function in a chimeric transcription factor. Basic research and genome sequencing efforts may reveal new proteins and homologs of existing repressors that could also be evaluated in this assay system.

Clearly, much information is required in order to define an effective strategy for modifying gene transcription in plants and there is a need to develop a rapid assay to test these various parameters. This study describes the development of a transient assay in plant cells to test the function of effector proteins on the transcription of target reporter genes. Different effectors and reporter configurations were tested in this assay to investigate the behavior of these novel zinc finger transcription factors and to develop a strategy for targeted gene regulation in plants. In this study, reporter target genes transiently transfected as a plasmid into plant cells were shown to respond to effector proteins in a similar manner to target genes integrated into chromosomal DNA. Two recent reports (Guan et al., 2002; Ordiz et al., 2002) demonstrated the stable, heritable regulation of endogenous plant genes by ZFP transcription factors and found that transient expression of effector proteins provides similar regulation as stable expression. Combined with the experiments described here, these results support the idea that effector regulatory function observed in this transient assay are predictive of the behavior of the ZFP transcription factor in stably transformed plants.

Continuing efforts using transient assays like the one developed in this study will identify the regulatory domains and effector designs that provide the optimal combination of activity, specificity, position and promoter dependence. Combined with inducible or tissue specific effector expression, this technique could provide highly precise control over the expression of a target gene. The results of these experiments indicate that this technology will be a powerful tool for basic plant research and agricultural biotechnology.

**Experimental Procedures**

**Reporter constructs**

The promoter from CsVMV (Verdaguer et al., 1998; Verdaguer et al., 1998) and its derivatives were used to express a reporter gene encoding the luciferase enzyme. Six copies of the binding site (6 × 2C7) for the C7 ZFP were inserted at various locations within the promoter. Plasmid pAluc contains the full length CsVMV promoter with no C7 binding sites. p5'C7A has the 6 × 2C7 binding site at the 5' end of the full-length CsVMV promoter. p3'C7A has the 6 × 2C7 binding site at the 3' end of the full length CsVMV promoter near the start of translation for the luciferase gene. pc7'AE contains the full length CsVMV promoter with 6 × 2C7 binding site replacing nucleotides −112 to −63. The 6 × 2C7 binding site was placed at the 5' end of CsVMV promoter fragments of different lengths to generate p5'C7C (−222 to +72), p5'C7D (−178 to −72), and p5'C7F (−112 to +72). prbTATA is a minimal promoter with a TATA box but without a ZFP binding site (actagtgctacgctgcagtaataaatgctgcatctccgttgtgtggaaacccacagt). pC7rbTATA is identical to prbTATA except that there is a 6 × 2C7 binding site at the 5' end of the minimal promoter. pCAT-RL was constructed as a control, which contains the 35S promoter driving expression of RL.

**Activator and repressor constructs**

Effector proteins were constructed in the same manner used by Beerli et al. (1998) and blunt end cloned into a TMV expression vector under the control of the coat protein subgenomic promoter (Padgett et al., 1996). Plasmid p2C7-SID consists of the 6-finger 2C7 ZFP fused to the C-terminus of the Sin3 interaction domain (SID, amino acid sequence of 1−36) (Ayer et al., 1996). Plasmid p2C7-KRAB contains the 6-finger 2C7 ZFP fused to the C-terminus KRAB domain (amino acid sequence of 1−97) (Margolin et al., 1994). Plasmid p2C7-VP64 contains the 6-finger 2C7 ZFP fused to the N-terminus of four tandem repeats of the minimal VP16 activation domain (VP64) (Beerli et al., 1998). Plasmid pC7-VP64 is 3-finger C7 ZFP fused to the VP64 activation domain. The control vector pC7-GFP contains the 3-finger ZFP C7 fused to the green fluorescent protein (GFP). The 2C7-VP64 coding sequence was also cloned 5’ of the maize ZmUbi (Cornejo et al., 1993) promoter for expression in maize protoplasts.

**Tobacco BY2 and maize HE89 cell line maintenance**

Cell lines were maintained at 28°C in the dark in shaking flasks with MS-based tissue culture medium (2.2 g l−1 Murashige and
Skoog Plant Salt Base - Gibco BRL #11117-074, 0.1 g l⁻¹ myo-inositol, 1 mg l⁻¹ thiamin-HCl, 0.2 mg l⁻¹ 2,4-D, 10 g l⁻¹ sucrose, 0.4 M mannitol, pH 5.8) and subcultured about every 7 days.

Transient transfection of BY2 and HE89 protoplasts

BY2 cells were transfected with purified DNA or RNA by electroporation essentially as described by Watanabe et al. (1987) and incubated at 28°C in dark for 24–72 h. Two reactions were performed for each treatment. Cells were collected by centrifugation and lysed by a freeze-thaw method in 80 μl of 1.2x Passive Lysis Buffer (Promega). HE89 protoplasts were prepared using methods similar to those for BY-2 cells and were transfected by PEG precipitation (Chourey and Zurawski, 1981). About half million protoplasts were used for each treatment. Ten micrograms plasmid DNA or viral RNA was used in transient transfection assays of tobacco BY-2 protoplasts.

Viral RNA transcription

The effector proteins pC7-GFP, p2C7-SID, p2C7-KRAB, pC7-VP64, and p2C7-VP64 were cloned into a TMV expression plasmid (Padgett et al., 1996). One microgram of plasmid DNA was digested with KpnI and cleaned with phenol:chloroform or via Qiaquick PCR purification (Qiagen). RNA was transcribed from the template DNA with the T7 Megascript Kit (Ambion). The concentration of resulting RNA was adjusted to 4 μg ml⁻¹ with MOPS (10 mM, pH 7.0) and stored at −80°C.

Luciferase assay

The luciferase assay was performed using 20 μl of cell lysate with 100 μl of luciferin assay solution (Promega) using an EG&G Wallac luminometer with a 4-sec delay and a 15-sec integration of emitted light. In the dual luciferase assays, 20 μl of cell lysate was assayed for LUC and RL using the Dual Luciferase System (Promega). One reaction was used with KpnI and cleaned with phenol:chloroform or via Qiaquick PCR purification (Qiagen). RNA was transcribed from the template DNA with the T7 Megascript Kit (Ambion). The concentration of resulting RNA was adjusted to 4 μg ml⁻¹ with MOPS (10 mM, pH 7.0) and stored at −80°C.

Generation of stable reporter cell lines

BY2 protoplasts were prepared for electroporation as described above and 2 x 10⁵ cells were electroporated with 1–10 μg of the pBin19 (Bevan, 1984) binary transformation vector (pbinC7AE). Protoplasts were incubated at 28°C in 15 ml protoplast culture medium (Watanabe et al., 1987) for 48 h. Cells were collected by centrifugation at 300 g for 2 min and plated on BY-2 media (2.2 g l⁻¹ Murashige and Skoog Plant Salt Base - Gibco BRL #11117-074, 0.1 g l⁻¹ myo-inositol, 1 mg l⁻¹ thiamin-HCl, 0.2 mg l⁻¹ 2,4-D, 10 g l⁻¹ sucrose, 0.4 M mannitol, 0.6% Agar, pH 5.8) containing 50 μg ml⁻¹ kan and incubated at 28°C in dark. Clumps of transformed cells were visible in 3–4 weeks and were transferred to fresh plates. When clumps were sufficiently large, they were assayed for luciferase activity and for the presence of plasmid integration by PCR. Suspension cell cultures were developed from positive events for use in transient assays.

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