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Functional characterization of the geminiviral conserved late element (CLE) in uninfected tobacco

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Abstract

The conserved late element (CLE) was originally identified as an evolutionarily conserved DNA sequence present in geminiviral intergenic regions. CLE has subsequently been observed in promoter sequences of bacterial (T-DNA) and plant origin, suggesting a role in plant and plant viral gene regulation. Synthetic DNA cassettes harboring direct repeats of the CLE motif were placed upstream from a −46 to +1 minimal CaMV 35S promoter-luciferase reporter gene and reporter activity characterized in *Nicotiana* species during both transient and stable expression. A single direct-repeat cassette of the element (2 × CLE) enhances luciferase activity by 2-fold, independent of the element's orientation, while multiple copies of the cassette (4–12 × CLE) increases activity up to 10- to 15-fold in an additive manner. Transgenic tobacco lines containing synthetic CLE promoter constructs enhance luciferase expression in leaf, cotyledon and stem tissues, but to a lesser extent in roots. Single nucleotide substitution at six of eight positions within the CLE consensus (GTGGTCCC) eliminates CLE enhancer-like activity. It has been previously reported that CLE interacts with the AC2 protein from Pepper Huasteco Virus (PHV-AC2). PHV-AC2 (also called AL2 or C2) is a member of the transcriptional activator protein, or TrAP, gene family. In transient and stable expression systems PHV-AC2 expression was found to result in a 2-fold increase in luciferase activity, irrespective of the presence of CLE consensus sequences within the reporter's promoter. These data suggests that the PHV-AC2 protein, instead of interacting directly with CLE, functions as either a general transcriptional activator and/or a suppressor of post-transcriptional gene silencing.

Abbreviations: AC2, geminivirus encoded TrAP protein; Ags^t, agropine synthase terminator; *AYVV*, Ageratum yellow veinvirus; *bar*, phosphinothricin acetyl transferase; BGMV, Bean golden mosaic virus; CaMV, Cauliflower mosaic virus; CaMV35S, Cauliflower mosaic virus 35S promoter; CLCuV, Cotton leaf curl virus; DTT, dithiothreitol; HcPro, viral helper component Protein (suppressor of silencing); GUSi, intron-modified β-glucuronidase; IM, infiltration media; Km, kanamycin; LB, Luria–Bertani; FiLUC, firefly (*Photinus pyralis*) intron-modified luciferase; MES, 2-(*N*-Morpholino)ethanesulfonic acid; MiMV, Mirabilis mosaic virus; MS, Murashige and Skoog; Nos^t, nopaline synthase terminator; *Npt II*, neomycin phosphotransferase II; pAg7, gene 7 terminator; pBS, pBluescript II (SK+); PClSV, peanut chlorotic streak virus (promoter); PGMV, Pepper golden mosaic virus (formally Serrano golden mosaic virus); PHV, Pepper Huasteco virus; Pnos, nopaline synthase promoter; PTGS, post-transcriptional gene silencing; PVY, Potato virus Y; R_0, primary transformant; R_1, progeny of self fertilized R_0 plant; RiLUC, *Renilla reniformis* (sea pansy) intron-modified luciferase; RLU, relative light units; TBE, Tris–Borate EDTA; T-DNA, transferred-DNA; TGMV, Tomato golden mosaic virus; ToLCV, Tomato leaf curl virus; TYLCV, Tomato yellow leaf curl virus; TrAP, transcription activating protein; $35S^t$, CaMV 35S terminator

Introduction

A screen of directly repeated (DR) DNA sequence elements from the intergenic regions of 28 sequenced geminiviruses and nanoviruses identified five DR motifs that both display enhancer-like activity, and contain a DNA segment termed the "conserved late element" or CLE (Velten *et al.*, 2005). The CLE motif had initially been identified in geminiviral DNA sequences by comparative genome analysis (Arguello-Astorga *et al.*, 1994). The CLE consensus sequence (GTGGTCCC) is enriched within geminiviral entries in GenBank (~17-fold relative to random short sequences of identical base composition) and within the *Arabidopsis* genome database (~4-fold enhanced in promoter sequences {−01 to −500 bp, relative to translation start} compared to protein coding sequences) (Velten *et al.*, 2005). Single copies of a CLE-like motif have also been identified within close proximity ($\leq$ 250 bp) of the predicted TATA box binding site from viral (CaMV {35S}, FMV {34S}, MiMV and CLCuV), bacterial (T-DNA, octopine synthase) and plant (e.g. *GapC4*, *PCNA*) promoters, suggesting CLE functions in viral and plant gene regulation (literature summarized in Table 1).

Direct repeats of CLE-like motifs fused to minimal (−46 to +8 bp) or truncated (−90 to +1 bp) CaMV 35S promoters (CLE-46 and CLE-90, respectively) have been shown to enhance reporter gene (GUS) expression in bombarded pea and tobacco leaf tissues (Ruiz-Medrano *et al.*, 1999). Furthermore, transformed tobacco plants harboring the CLE-90::GUS fusion present a GUS expression pattern different from that observed in CLE-lacking control transgenics (−90::GUS) (Ruiz-Medrano *et al.*, 1999). Kosugi and colleagues also demonstrated that a head-to-tail dimer of the CLE-like motif, IIa, (Table 1) inserted upstream of a minimal (−54 to +5 bp) CaMV 35S-GUS fusion could stimulate GUS activity by ~5.5-fold compared to a CLE-minus control construct in tobacco protoplasts (Kosugi *et al.*, 1995). CLE elements derived from geminiviruses added to minimal (−46 to +1 bp) CaMV 35S promoter-luciferase gene constructs also show a positive effect (~2-fold) on expression of the luciferase reporter gene (Velten *et al.*, 2005).

Several recent reports (Table 1) have described plant DNA binding proteins that associate with a CLE-like DNA sequence present in many plant promoter regions. The CLE-like sequence element IIa, GTGGGCCCGT, identified in the promoter of the rice proliferating cell nuclear antigen (PCNA) gene (Kosugi *et al.*, 1995), contains a core sequence (GGNCCC) which is necessary for the binding of rice (PCF1-6) (Kosugi and Ohashi 2002) and maize (TBP1) (Doebley *et al.*, 1997) transcription factors containing the TCP DNA binding domain (Cubas *et al.*, 1999). A similar sequence element (tccgGTGGGCCCGaaac) identified from the maize *GapC4* promoter also contains a CLE-like sequence and can bind tobacco nuclear extracts (Geffers *et al.*, 2000). These reports indicate that there are plant *cis*-acting promoter elements closely resembling the CLE consensus that are capable of interacting with plant DNA binding proteins implicated in gene regulation.

It has been reported that the CLE sequence element upstream from the Pepper Huasteco Virus (PHV) coat protein gene is involved in PHV-AC2 mediated transactivation of coat gene expression (Ruiz-Medrano *et al.*, 1999). Furthermore, the prevalence of CLE elements in three major dicot-geminivirus lineages correlates well with observed conservation in the amino acid sequences of a putative DNA-binding domain within the corresponding viral AL2 genes, homologs of the PHV-AC2 gene (Arguello-Astorga *et al.*, 1994). These reports suggest that CLE might be a functional target of AC2 (or AL2), however, it has recently been demonstrated that mutation of a CLE consensus sequence upstream from the TGMV coat protein does not affect activation by TGMV-AL2; neither in plants containing replicating viral genomes nor in *Nicotiana benthamiana* protoplasts transiently expressing a plasmid construct (Sunter and Bisaro, 2003). Additionally, it has been shown that the late promoters of *Bean golden mosaic virus* (BGMV), which lack any CLE motif, are functionally equivalent to the TGMV late promoters and can be activated by homologous or heterologous TrAP proteins (TGMV-AL2 or BGMV-AL2) (Hung and Petty, 2001). There is clearly some question as to exactly how AC2 and homologous proteins from different geminiviruses interact with host and virus systems to affect viral (and possibly host) gene expression.

Transient gene expression systems have been shown to trigger post-transcriptional gene

Table 1. Review of CLE-like element identification and analysis.

Mode of CLE identification	Source organism	Accession #	Comments	Test organism (method)	CLE effect	Reference	CLE sequence alignment (underlined = consensus {GTGGTCCC})
Sequence analysis	Geminiviruses	–	Sequence comparison of the C region of EH geminiviruses defines CLE	*In silico*	–	Arguello-Astorga *et al.* (1994)	(A/G)(A/T)GTGGTCCC
	Arabidopsis	–	CLE consensus enriched in Arabidopsis promoters	*In silico*	–	Velten *et al.* (2005)	GTGGNCCC
Plant/viral trans-factors and promoters	Pepper Huasteco [B]	X70420	AC2/TrAP transactivates promoter (coat protein) constructs harboring CLE	*N. bentamiana* & Pea (particle bombardment)	Transcriptional enhancer	Ruiz-Medrano *et al.* (1999)	GTGGTCCC
	Tomato golden mosaic [A]	NC001507	AL2/TrAP fails to transactive promoter (coat protein) containing CLE	*N. bentamiana* (protoplasts)	–	Sunter *et al.* (2003)	GTGGTCCC
	Bean golden mosaic [A]	NC001439	AL2 transactivation of promoters (AT1 & BR1) that lack CLE	*N. bentamiana* (protoplasts)	–	Hung and Petty (2001)	–
	Rice/Maize (DNA binding proteins)	–	TCP DNA binding proteins, PCF1-6 & TB1, bind CLE-like DNA seqeunces	*In vitro* (gel mobility shift)	–	Kosugi and Ohashi (2002)	GTGGBCCC (class II) GGNCCC (class I)
	Arabidopsis (TCP targeted promoters)	–	Mutations in CLE-like elements can lower Arabidopsis promoter activity	*N. tabacum*-Sumsun NN (transgenic callus)	–		
	Rice (CLE-like class I & II)	–	Class I CLE-like elements can enhance promoter function	*N. tabacum*-Sumsun NN (transgenic callus)	Enhancer (class I)		
	Rice	X54046	CLE-like elements IIa & IIb required for meristem specific activity	*N. bentamiana* (protoplasts)	Enhancer-Iia (~5.5 fold*)	Kosugi *et al.* (1995, 1997)	GTGGGCCCGT (IIa) ATGGTCCCAC (IIb)
	Maize	L40803	CLE-like Binding site for tobacco nuclear extracts (*GapC4*)	*In vitro* (Gel mobility shift)	–	Geffer *et al.* (2000)	tccgGTGGGCCCGaaac
Viral elements	Ageratum yellow vein virus [A]	X74516	Transcriptional enhancer (DR40)	*N. tabacum*-SR1 (Agro-infusion)	Enhancer (~1.61 fold*)	Velten *et al.* (2005)	TACGTGGTCCCC^ TACGTAGTCTCC
	Leonurus mosaic virus [A]	U92532	Transcriptional enhancer (DR02)	*N. tabacum*-SR1 (Agro-infusion)	Enhancer (~1.72 fold*)	Velten *et al.* (2005)	CGTGGTCCCT^ CGTGGTCCCT
	Leonurus mosaic virus [A]	U92532	Transcriptional enhancer (DR21)	*N. tabacum*-SR1 (Agro-infusion)	Enhancer (~1.95 fold*)	Velten *et al.* (2005)	CGTGGTCCCT^ CGTGGTCCCT*
	Pepper golden mosaic virus [A]	U57457	Transcriptional enhancer (DR17)	*N. tabacum*-SR1 (Agro-infusion)	Enhancer (~2.16 fold*)	Velten *et al.* (2005)	GTGGTCCCT^ GTGGTCCCA*
	Pepper Huasteco virus [B]	X70420	Transcriptional enhancer (DR33)	*N. tabacum*-SR1 (Agro-infusion)	Enhancer (~1.86 fold*)	Velten *et al.* (2005)	GTGGTCCCAAAGGAC^ GTGGTCCCAAATGAC*
	Tomato leaf curl virus	U38239	Transcriptional enhancer (DR37)	*N. tabacum*-SR1 (Agro-infusion)	Enhancer (~2.03 fold*)	Velten *et al.* (2005)	TTTTGTGGGCCCT^ TTTTGTGGTCCCT

Table 1. Continued

Mode of CLE identification	Source organism	Accession #	Comments	Test organism (method)	CLE effect	Reference	CLE sequence alignment (underlined = consensus {GTGGTCCC})
	Cauliflower mosaic virus {35S}	V00141	Not characterized	–	–	Benfey Chua (1990)	gacaGTGGTCCCaaag
	Figwort mosaic virus {34S}	X16673	Not characterized	–	–	Sanger *et al.* (1990)	tggGTGGTCCCcac
	Mirabilis mosaic virus	NC004036	Not characterized	–	–	Dey Maiti (1999)	tccaGTGGTCCCtcca
	Cotton leaf curl virus	AY312430	Not characterized	–	–	Xie *et al.* (2003)	tttGTGGGCCCccg
Bacterial (T-DNA)	Octopine synthase	AF242881	Not characterized	–	–	Leisner and Gelvin (1989)	TttgGTGGACCCttgag

Key: [], A or B genome and { }promoter transcript size.
–, not tested.
*, minimal 35S promoter = 1.0.
^, 10 bp spacer sequence (GAAGATAATC).
**, inverse orientation.

silencing (PTGS) (Johansen and Carrington, 2001; Voinnet *et al*., 2003; Cazzonelli and Velten, 2004) and recently it was demonstrated that AC2-like proteins can act as viral suppressors of PTGS (Voinnet *et al*., 1999; van Wezel *et al*., 2002; van Wezel *et al*., 2003; Selth *et al*., 2004; Vanitharani *et al*., 2004). Since previous studies of the trans-activation effects of PHV-AC2 on CLE-containing promoters were performed in transient expression systems, it is possible that PHV-AC2 does not directly interact with the target genes, but instead may increase gene expression by suppressing PTGS occurring within the test system. To further complicate the field, it has recently been reported that geminiviral proteins (eg AC2, AC4) can play different roles in mediating viral synergism and suppression of PTGS (Vanitharani *et al*., 2004), presenting the possibility that the CLE sequences may still be capable of interacting with TrAP proteins indirectly through unknown intermediates.

In this report, the *in vivo* properties of single, multiple and mutated direct-repeat units of the CLE consensus (fused to a −46 to +1 minimal CaMV 35S promoter) have been quantified in tobacco leaf tissues using luciferase as a reporter of promoter function. The whole-plant expression characteristics of functional synthetic promoters containing multiple copies of the CLE motif (up to six direct repeats) are described. In addition, the effects of the *PHV*-AC2 protein on CLE-containing, and CLE-lacking, promoter function is explored in transient and stable gene expression systems. These data address the role of AC2 as a possible suppressor of PTGS, or general activator of transcription regulation, and support the significance of the CLE motif in viral and plant gene regulation.

Methods

Construction of synthetic promoter–reporter gene cassettes

Plasmid constructs were prepared using standard cloning techniques (Sambrook and Russell, 2001). The normalization vector, pE1778-SUPER-RiLUC was prepared as previously described (Cazzonelli and Velten, 2003). pE1778-SUPER-RiLUC harbors a castor bean

catalase intron-modified *Renilla reniformis* luciferase reporter gene (RiLUC), controlled by the synthetic super promoter (Ni *et al*., 1995) present within the binary vector, pE1778. The pE1778-based plasmid contains a plant-functional kanamycin resistance marker consisting of the neomycin phosphotransferase II coding region controlled by the nopaline synthase promoter and the T-DNA gene 7 terminator (Becker *et al*., 1992) (S. Gelvin, personal communication).

Plasmids used to test promoter element function (pTest, pTm35, pTm35enh and pTPGEL1; Figure 1A–D) contain two gene fusions cloned head to tail within the multiple cloning region of the binary vector, pPZP200 (Hajdukiewicz *et al*., 1994). All 'test' plasmids contain a marker gene for selection of transgenic plants positioned near the T-DNA left border. The selectable marker consists of the *bar* open reading frame, encoding phosphinothricin acetyl transferase (Accession number: AX235900), controlled by a peanut chlorotic streak virus promoter (-240 to +1 bp) (Maiti and Shepherd, 1998) and a CaMV 35S transcription termination signal. The second gene fusion within the test plasmids consists of an intron-modified firefly luciferase gene (Mankin *et al*., 1997) with a small 5′ multiple cloning region for inserting promoter regulatory regions to be tested. The firefly luciferase gene is fused to the nopaline synthase transcription terminator.

The suite of test (pT*) plasmids vary in the transcription control sequences present 5′ to the firefly luciferase gene: ''pTest'' (Figure 1A) contains no plant transcription regulatory components; ''pTm35''(Figure 1B) contains a minimal (−46 to +1 bp) 35S promoter (Benfey and Chua, 1990); ''pTm35enh'' (Figure 1C) has a single enhancer element (−299 to −99 bp) from

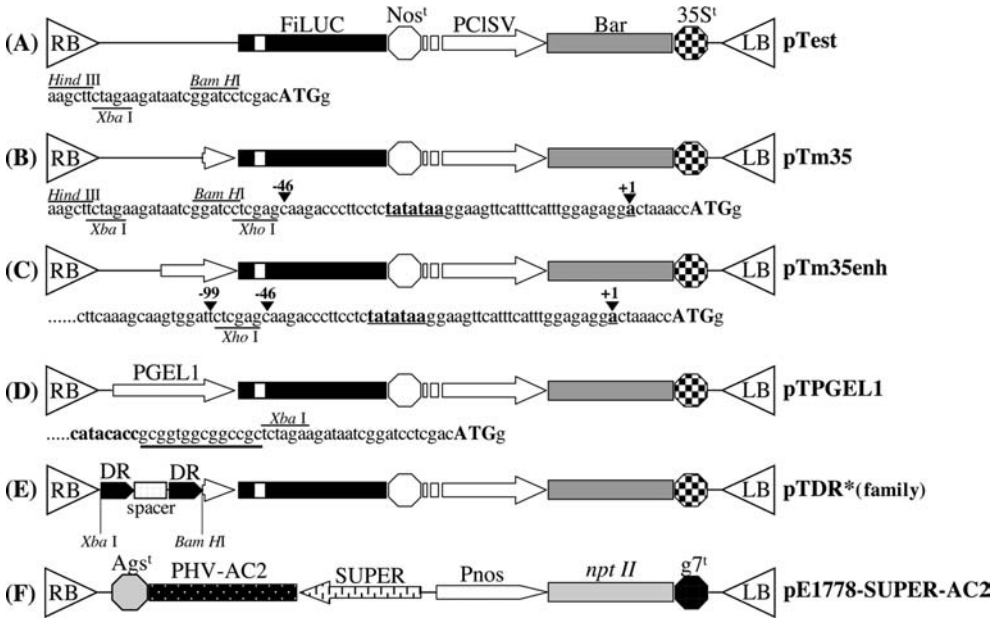

Figure 1. Schematic diagram describing binary vectors. A–E: test vectors (pT*); T-DNA right border (RB); Firefly-intron coding region (FiLUC); nopaline synthase terminator (Nos^t); peanut chlorotic streak virus promoter (PClSV); phosphinothricin acetyl transferase (Bar); 35S terminator ($35S^t$); T-DNA left border (LB). (A) pTest: promoterless-FiLUC. (B) pTm35: minimal CaMV 35S promoter (−46 to +1 bp from CaMV 35S promoter). (C) pTm35enh: CaMV 35S enhancer region (−299 to −99 bp) added to pTm35; (D) pTPGEL1: segment of the PGEL1 promoter (−1193 to +85 bp) fused to FiLUC gene. Lower case sequences highlighted in bold font indicate the 3′ end (untranslated leader region) of the PGEL1 promoter and underlined nucleotides show a GC rich sequence introduced during the cloning process. (E) pTDR*, general features of binary vectors containing direct-repeat CLE sequences (DR) separated by a 10-bp spacer. (F) pE1778-SUPER-AC2: Agropine synthase terminator (Ags^t); PHV-AC2 coding region (PHV-AC2); super promoter (SUPER); nopaline synthase promoter (Pnos); neomycin phospho-transferase II coding region (nptII); T-DNA gene 7 terminator ($g7^t$). pE1778-SUPER-AC2 is a PHV-AC2 expression vector used for co-expression of PHV-AC2. Sequences displayed below selected construct diagrams show the junction between the luciferase reporter gene and test promoter. The FiLUC start codon is in bold uppercase font. The TATA box is underlined and highlighted in bold font. The start of transcription underlined and highlighted in bold.

the CaMV 35S promoter (Benfey and Chua, 1990) added to the pTm35 minimal promoter using HindIII and XhoI; and "pTPGEL1" (Figure 1D) contains a segment (−1193 to +85) from the strong constitutive PGEL1 promoter (Accession number: AY819645) that was removed from pPGEL1::iGUS plasmid (Cazzonelli *et al.*, 2005) with *Xba*I and inserted into pTest (*Xba*I digested).

pTm35 (Figure 1B) plasmid served as a starting point to create a suite of synthetic cassettes that test the enhancer-like activity of single, multiple or mutated copies of CLE DR cassettes (pTDR*; Figure 1E, pTCLE*; Figure 4). PCR was used to amplify oligonucleotides that overlap by a 10 bp randomized stuffer sequence (GAAGATAATC) and are flanked by *Xba*I and/or *Bam*HI restriction endonucleases (Table 2). The resulting PCR products (CLE-spacer-CLE) were digested with the appropriate restriction enzyme(s) and cloned into pTm35 (digested with *Xba*I and/or *Bam*HI). A single direct-repeat cassette of DR#2, #17 and #33 (Table 1) was amplified using the appropriate primers (DR-Ux and DR-Lb; Table 2) and cloned into pTm35 in their original orientation creating the vectors pTDR#2(>), pTDR#17(<) and pTDR#33(<), respectively (Figure 1; (Velten, *et al.* 2005)). Multimerization of DR#2, #17 and #33 was accomplished using specific oligonucleotides (DR-Ux and DR-Lx; Table 2) and PCR to amplify a product that was subsequently cloned (in their original orientation as defined by the GenBank sequence file) into pTDR#2(>), pTDR#17(<) and pTDR#33(<), respectively. The resulting enhancer cassette multimers, pTDR#2–4 (>>), pTDR#2-L (>>>>>>), pTDR#17–5 (<<), pTDR#17-H (<<<<<), pTDR#33-7 (<<), pTDR#33-J (<<<<<) were sequenced in order to confirm that the orientation of the CLE direct-repeats were maintained during ligation. Each (>) indicates one copy of a CLE directly repeated dimer and describes the orienta-

Table 2. Primer sequences used during binary vector construction.

Primer	Sequence (underlined = CLE sequence, bold = mutation site)
DR2-Ux	5′ GCTCTAGACGTGGTCCCTGAAGATAATC 3′
DR2-Lb	3′ CTTCTATTAGGCACCAGGGACCTAGGGC 5′
DR2-Lx	3′ CTTCTATTAGGCACCAGGGAAGATCTCG 5′
DR17-Ux	5′ GCTCTAGAAGGGGACCACGAAGATAATC 3′
DR17-Lb	3′ CTTCTATTAGTCCCCTGGTGCCTAGGGC 5′
DR17-Lx	3′ CTTCTATTAGTCCCCTGGTGAGATCTCG 5′
DR33-Ux	5′ GCTCTAGAGTCATTTGGGACCACGAAGATAATC 3′
DR33-Lb	3′ CTTCTATTAGCAGGAAACCCTGGTGCCTAGGGC 5′
DR33-Lx	3′ CTTCTATTAGCAGGAAACCCTGGTGAGATCTCG 5′
CLE-Ux	5′ GCTCTAGAGTGGTCCCGAAGATAATC 3′
CLE-Lb	3′ CTTCTATTAGCACCAGGGCCTAGGGC 5′
CLEm1-Ux	5′ GCTCTAGA**A**TGGTCCCGAAGATAATC 3′
CLEm1-Lb	3′ CTTCTATTAG**T**ACCAGGGCCTAGGGC 5′
CLEm2-Ux	5′ GCTCTAGA**G**AGGTCCCGAAGATAATC 3′
CLEm2-Lb	3′ CTTCTATTAGC**T**CCAGGGCCTAGGGC 5′
CLEm3-Ux	5′ GCTCTAGA**G**TA**G**TCCCGAAGATAATC 3′
CLEm3-Lb	3′ CTTCTATTAGCA**T**CAGGGCCTAGGGC 5′
CLEm4-Ux	5′ GCTCTAGA**G**TG**A**TCCCGAAGATAATC 3′
CLEm4-Lb	3′ CTTCTATTAGCAC**T**AGGGCCTAGGGC 5′
CLEm5-Ux	5′ GCTCTAGA**G**TGG**A**CCCGAAGATAATC 3′
CLEm5-Lb	3′ CTTCTATTAGCACC**T**GGGCCTAGGGC 5′
CLEm6-Ux	5′ GCTCTAGA**G**TGGT**A**CCGAAGATAATC 3′
CLEm6-Lb	3′ CTTCTATTAGCACCA**T**GGCCTAGGGC 5′
CLEm7-Ux	5′ GCTCTAGA**G**TGGTC**A**CGAAGATAATC 3′
CLEm7-Lb	3′ CTTCTATTAGCACCAG**T**GCCTAGGGC 5′
CLEm8-Ux	5′ GCTCTAGA**G**TGGTCC**A**GAAGATAATC 3′
CLEm8-Lb	3′ CTTCTATTAGCACCAGG**T**CCTAGGGC 5′
AC2-5s	5′ CGTGTCGACATGGATTTACGCACC 3′
AC2-3′	5′ AGCTCTAGACTATTTAAGTAAATCTGCCCAGAAATCG 3′

tion (> or <) of the CLE motif relative to the promoter (>). pTCLE (Figure 4) was prepared by cloning a PCR product (amplified using CLE-Lx and CLE-Ub primers) into pTm35 (digested with *Xba*I and *Bam*HI). Mutagenesis of the CLE consensus (GTGGTCCC) elements within each mutant dimer was accomplished by substituting adenosine at the nucleotide position indicated (Table 2, Figure 4). Plasmids pTCLEm1 to pTCLEm8 (Figure 4) were constructed using specific primers (CLEm(-Ux and CLEm(-Lb) to PCR amplify a product containing two mutant copies of CLE for subsequent cloning into pTm35 (digested with *Xba*I and *Bam*HI).

Analysis of the ''helper component protein'' (HcPro) and AC2 for activity as suppressors of post-transcriptional gene silencing involved introduction of plasmids engineered to express these genes into Agrobacteria that were grown and mixed with lines expressing the various reporter constructs prior to leaf infiltration. The pBIN61-HcPro plasmid was used to express an HcPro gene from Potato virus Y (PVY) (Accession number: AY775290) under control of the CaMV 35S promoter (Voinnet *et al*., 2003). pE1778-AC2 contains the TrAP protein (AC2) from the PHV-A genome under control by the synthetic super promoter (Ni *et al*., 1995) (GeneBank accession number: AY044162, a plasmid clone of the PHV-A genome, pBluescript::*PHV*-A, was graciously provided by Dr. Robert Gilbertson). Primers AC2-5′ and AC2-3′ (Table 2) were used to PCR amplify the PHV AC2 coding region from the plasmid, pBluescript::PHV-A. The AC2 PCR product was purified, digested with SalI and XbaI, and cloned into pE1778 (Figure 1F). Subsequent sequencing of the pE1778-AC2 insert confirmed that the AC2 coding sequence exactly matched that used by Ruiz-Mendrano and coworkers (Ruiz-Medrano *et al*., 1999). The pIG121 plasmid, a CaMV 35S::GUS-intron binary vector, was used as a negative (non-suppressing) control in mixed Agrobacteria cultures, since it does not produce any protein product (suppressor or activator) that can directly impact Firefly-intron luciferase gene (FiLUC) activity. pIG121 is based upon the pBI101 binary vector and its detailed structure is described elsewhere (Akama *et al*., 1992). Predicted DNA sequence files for all the plasmids used in this research are available from the authors upon request.

Agrobacteria infiltration of tobacco leaf tissues

Plasmid vectors were electroporated into Agrobacteria (EHA105) as described (Walkerpeach and Velten, 1994). The pE1778-RiLUC (SR) line of Agrobacteria was further transformed with compatible test plasmids allowing RiLUC activity to serve as a normalization control between independent infusions. Agrobacteria lines were grown as individual cultures at 28°C in LB media containing the appropriate antibiotic selection (25 μg/ml kanamycin sulfate or 100 μg/ml spectinomycin) until each culture reached an OD_{600} of 0.8. The resulting cultures were separately centrifuged at $8000 \times g$ for 15 min, washed and re-suspended in an equal volume of infiltration media (50 mM MES (pH 5.6), 0.5% glucose, 2 mM $NaPO_4$, 100 μM acetosyringone). *Nicotiana tabacum* (SR1) and *N. benthamiana* plants were grown in hydroponic solution under artificial lighting with a 16/8 h photoperiod in an environmentally controlled growth room (22°C). Multiple individual leaves (2 leaves per construct) were mechanically infused with Agrobacteria by pressing the tip of the syringe against the lower surface of the leaf and applying gentle pressure to the plunger. Quantitative measurement of luciferase activity occurred 2–5 days post-inoculation (dpi) using an *in vivo* floating leaf-disk assay described below.

Stable transformation of tobacco

Transformation of tobacco (*N. tabacum*, cv SR1) was accomplished using Agrobacteria lines (EHA105) containing pTm35enh pTm35 (Figure 1) and the pTDR* family of binary vectors. Agrobacteria were co-cultivated with tobacco leaf discs as described previously (Horsch *et al*., 1988) (Svab *et al*., 1995). Regenerated transgenic plants were maintained on MS-agar medium containing B5 vitamins, 2.5 mg/l BASTA (phosphinothricin or glufosinate-ammonium salt, AGREVO) and 500 μg/ml Claforan (sodium cefotaxime, Hoechst). Primary transformants were rooted on selective media, transferred to seedling soil mixture and maintained under humid conditions for a week prior to being potted to a sandy/peat mixture and grown in an environmentally controlled glasshouse (standard conditions = 70% humidity, 22°C, 16/8 h photoperiod). First generation

selfed seed (R_1) were harvested, germinated on herbicide treated soil (100 μl/l {v/v} of Agrevo) and grown as above for 4 weeks (transformation confirmed by luciferase assay). Generation of transformed tobacco (*N. tabacum*, cv Ti68) containing pPZP2.5LuNt (PGEL1 promoter::Firefly Luciferase, lines (3-6, 4-10) and pPZP35sLuNt (CaMV 35S promoter::Firefly Luciferase, lines (4-5, 10-6) was described previously (Cazzonelli *et al*. 2005).

Luciferase assays

Quantitative measurement of luciferase activity was achieved using an *in vivo* floating leaf-disk assay (Cazzonelli and Velten, manuscript in preparation) and/or the *in vitro* Dual-luciferase® kit from Promega Corp (*in vitro* assays were performed according to the manufacturers instructions). When both assays were applied, separate leaf disks from the same infusion were used for *in vivo* assays and *in vitro* assays. For each *in vivo* assay two leaves from separate plants were infused and four to eight leaf disks (3 mm diameter) per infusion carefully cut using a sharpened cork borer (excised leaf discs were manipulated very carefully to avoid additional wounding). Separate disks were floated on luciferase assay media (LAM: Luciferin Assay Media for FiLUC and CAM: Coelenterazine Assay Media for RiLUC) in different wells of a 96 well, white-walled, microtiter plate and light production measured over time using a FLUOstar Optima luminometer® from BMG Lab Technologies Inc. LAM buffer contains: 1 mM luciferin (diluted from a 100 mM stock dissolved in 20 mM MES, pH 5.6), 50 mM MES (pH 5.6), 0.5% glucose, 2 mM $NaPO_4$, 0.5% v/v DMSO and CAM buffer contains: 11.8 μM coelenterazine (diluted from a 2.36 mM stock dissolved in methanol), 25 mM glucose, 0.1 M $NaPO_4$ (pH 8), 0.05 M NaCl, 1 mM EDTA, 10 mM DTT. The peak of light emission over a 90 min time course was determined for each sample and referred to as the relative light units emitted (RLU). Background light emission in the firefly and sea pansy luciferase assays was determined by floating leaf discs from wild type SR1 tobacco on LAM and CAM media, respectively. Relative light units (RLU) were adjusted by subtracting background luminescence from the maximum levels of light emission determined for each sample. When indicated, firefly luciferase values from Agrobacteria infusions were normalized between test assays using Renilla luciferase activity generated by the compatible pE1778-SUPER-RiLUC binary plasmid.

CLE enhancer activity, transient assay

CLE enhancer activity in the transient expression system was measured by comparing firefly luciferase activity in *N. tabacum* leaves infused with Agrobacteria lines harboring the indicated test plasmid, to FiLUC expression driven by the CaMV 35S minimal promoter (pTm35). Leaf discs (n = 6–8) were collected at 2–3 time points post-infusion, and firefly (FiLUC) and sea pansy (RiLUC) luciferase activities measured using the *in vivo* assay or a combination of both *in vivo* and *in vitro* assays. The degree of enhancement was calculated as the ratio of luciferase activity in the test constructs to that from the control pTm35 infusion (RL_{UpT*}/RLU_{pTm35}).

CLE enhancer activity, stable transgenics

Primary (R_0) *N. tabacum* transgenics containing the pT* test constructs were selected for herbicide resistance and grown under standard conditions for 4 weeks prior to being assayed for luciferase activity using the *in vivo* floating leaf disc assay (two leaf discs from multiple independent transgenic line {pTDR* (n = 8–19 lines); pTm35 (n = 7 lines) and pTm35enh (n = 10 lines)}. Selfed seed from the R_0 plants (= Secondary, R_1 transformants) were germinated on herbicide treated soil and grown under standard conditions (see above) for four weeks prior to assay. For each test construct multiple independent transgenic lines were assayed {pTDR* (n = 6–14 lines); pTm35 (n = 28 lines) and pTm35enh (n = 27 lines)}. For each line, a single leaf disc from each of eight resistant plants was removed (3rd or 4th true leaf) and assayed for luciferase activity using the *in vivo* assay. Activity enhancement by CLE in the pT* plants is reported relative to transgenics containing the minimal promoter (pTm35) control, calculated as the ratio of luciferase activities (RL_{UpT*}/RLU_{pTm35}).

Whole tissue bioluminescence

Firefly luciferase activity was directly observed in intact plant tissues using a Photometrics Cool SNAP HQ imaging system (Roper Scientific).

Transgenic seeds (R_1) were germinated on a soil/peat mixture containing herbicide (100 μl/l (v/v) of Agrevo) and three week old seedlings (grown under standard conditions) were carefully dissected into leaf, cotyledon, stem and root tissues, using razor blades and broad forceps to minimize tissue damage. The excised tissues were submerged in an aqueous solution containing 0.4 mM luciferin and 100 mM sodium citrate (pH$\sim$5.6) and photon counting performed after 2 min of incubation (at ambient temperature in the dark). Exposures of 60, 300 and 600 s were taken for all transgenic lines examined. Image J software (v 1.32, available at <http://rsb.info.nih.gov/ij/>) was used to enhance different light levels and to create overlays of multiple images (threshold settings were maintained at 99–400 for all exposures).

PHV-AC2 activation of reporter activity, transient assay

pE1778-SUPER-AC2, an Agrobacteria line containing a binary plasmid engineered to express the AC2 protein from Pepper Huasteco Virus (PHV-AC2) under control of the super promoter, was co-infused into *N. tabacum* and *N. benthamiana* leaves (2 leaves per plant per assay) in a mixed culture containing an equal density of Agrobacteria harboring the indicated test construct (pTm35, pTDR#33, pTCLE, pTDR#2-L or pTPGEL1). Three to four leaf discs per mixed infusion were harvested at 90 h post-infusion and assayed for firefly luciferase activity using the *in vivo* assay. The luciferase enhancement ratio (RLU_{AC2}/RLU-pIG121) was determined by comparing RLU emitted from the AC2-containning infusions to values from control mixtures containing pIG121 (GUS expression plasmid) instead of the pE1778-SUPER-AC2 line.

PHV-AC2 activation of reporter activity, stable transgenics

One half of a leaf from transgenic *N. tabacum* transformed with the indicated luciferase construct (PGEL1 promoter::firefly luciferase, CaMV 35S promoter::firefly luciferase, pTm35enh or pTDR#2-L) was infiltrated with an Agrobacteria line containing either pE1778-SUPER-AC2 or pBIN61-HcPro (a confirmed viral suppressor of PTGS) and the other half of the leaf (separated by the mid-rib) with Agrobacteria containing the negative control construct (pIG121, GUS expression). Infusions were performed in duplicate using leaf tissues from two developmentally similar plants. Three to four leaf discs from each infusion were harvested 114 h post-infusion and assayed for luciferase activity using the *in vivo* assay. The luciferase enhancement ratio (RLU_{HcPro} or $_{AC2}$/RLU_{pIG121}) was determined by comparing the RLU emitted from the AC2 or HcPro infusions relative to the control (pIG121 infusion) for each plant tested. The average enhancement ratio from duplicate assays performed on 2 to 4 independent lines (pPZP2.5LuNt#3-6, 4-10; pPZP35sLuNt#4-5, 10-6; pTm35enh#3, 4, 5, 6; pTDR2-L#6, 12) was calculated (error bars represent standard error).

Results

Multiple copies of the CLE motif enhance luciferase activity in an additive manner

Directly repeated copies of several naturally occurring viral sequences containing the CLE motif (e.g. DR#2, DR#17 and DR#33; Table 1) had previously demonstrated the capacity to enhance ($\sim$2-fold) reporter gene activity when introduced upstream of a -46 to $+1$ minimal CaMV 35S promoter: FiLUC construct, independent of CLE orientation (Velten *et al.*, 2005). In order to determine if multiple copies of CLE direct repeats can further enhance transcription, a series of constructs (pTDR*, Figure 1) harboring two {pTDR#2-4(>>), pTDR#17-5(<<) and pTDR#33-7(<<)}, four {pTDR#17-H and pTDR#33-J(<<<<)} or six {pTDR#2-L(>>>>>>)} copies of each CLE containing viral direct-repeat cassette were generated from synthetic oligonucleotides (described in Velten *et al.*, 2005) based upon GenBank sequence files (sequence and accession numbers listed in Table 1). The resulting element cassettes were multimerized and fused to the minimal CaMV 35S::FiLUC reporter (each '>' or '<' represents a direct-repeat sequence cassette containing 2 copies of CLE, orientation is relative to the minimal 35S promoter {oriented rightward}). The resulting T-DNA binary plasmid constructs were tested for luciferase activity using both an Agrobacteria-infusion transient assay system developed for *N. tabacum* leaf tissues (Figure 2A) and in stably transformed plants

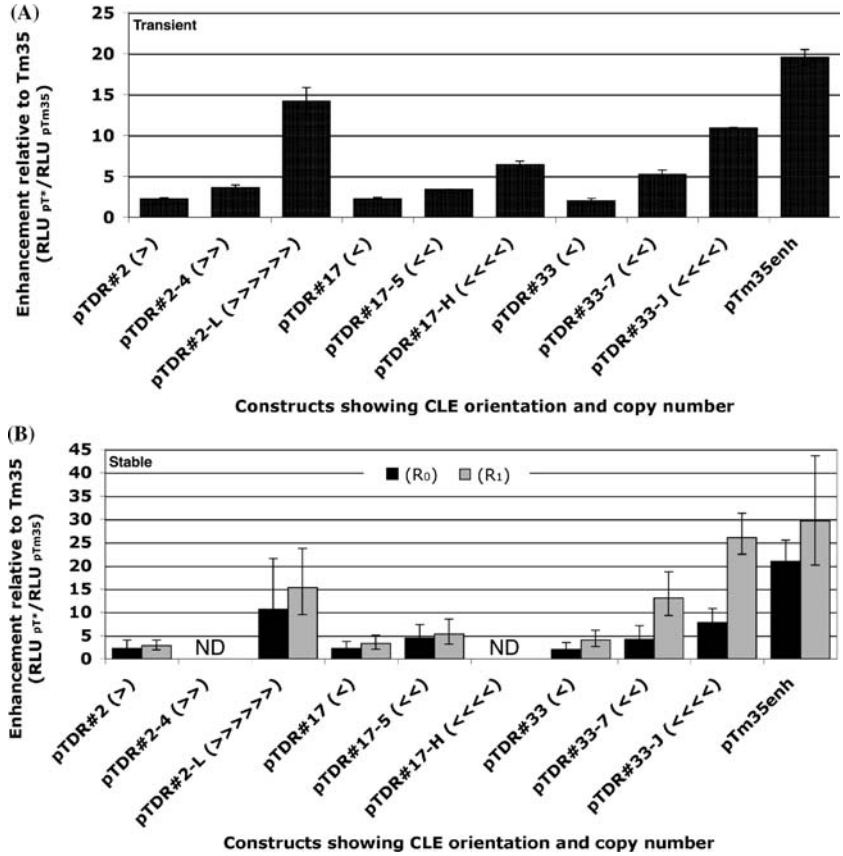

Figure 2. Quantification of CLE enhancer activity in tobacco leaf tissues. (A) Transient luciferase expression: *N. tabacum* leaves were infused with Agrobacteria lines harboring the indicated pT* test plasmid or the minimal promoter (pTm35) control construct. Firefly (FiLUC) and sea pansy (RiLUC) luciferase activities were measured using both *in vivo* and *in vitro* assays and results presented as the ratio of normalized firefly luciferase activities from test constructs over pTm35 controls (RLU_{pT*}/RLU_{pTm35}). The value shown for each test construct is the mean ratio from two time points (post infusion), each measured via both *in vivo* and *in vitro* assays (error bars represent standard error among assays). (B) Stable luciferase transgenics: Herbicide resistant primary (R_0) or first generation selfed (R_1) *N. tabacum* transgenics containing the indicated pT* test construct were assayed for luciferase activity using the *in vivo* assay. Activity enhancement is reported relative to minimal promoter pTm35 transgenic control lines, calculated as the ratio of luciferase activities (RLU_{pT*}/RLU_{pTm35}) (error bars represent standard error among independent transformed lines).

(Figure 2B). A clear positive correlation between the number of CLE copies within the promoter and the level of luciferase activity was apparent in both the transient and stable systems (Figures 2A and 2B). As CLE copy number increased {eg. pTDR(33(<) = 2xCLE, pTDR(33-7(<<) = 4 × CLE, and pTDR(33-J (<<<<<) = 8 × CLE}, measured luciferase activity was enhanced accordingly. The orientation of the CLE motif within the pTDR* constructs had no clear impact upon the degree of promoter enhancement {compare DR#2(>) with DR#17(<) or DR#33(<), Figure 2A and 2B}. Eight to twelve copies of the CLE element {pTDR(33-J(<<<<<) = 8× CLE and pTDR(2-L(>>>>>>) = 12× CLE} increased luciferase activity to levels close to that resulting from addition of the well defined CaMV 35S enhancer region to the minimal promoter (pTm35enh; Figure 2A).

CLE element expression enhancement is displayed throughout transgenic tobacco seedlings

The elevated luciferase activity associated with multiple copies of CLE allowed further characterization of the motif's ability to affect

expression throughout tobacco seedlings. Tobacco transformants (R_2 plants, mixed hemizygous and homozygous) harboring single {pTDR#17(<), #33(<) and #2(>)} or multiple (pTDR#17-5(<), #33-J(<<<<<) and #2-L(>>>>>>>)} cassettes of the CLE-containing viral direct repeat were grown under uniform conditions and excised tissues (leaf, root, cotyledon and stem) from 6 to 8 seedlings per transgenic line examined for bioluminescence using a CCD camera (Figure 3). Since individual transformed lines typically show a large variation in absolute expression levels, digital overlays of images (captured under identical conditions) from individual seedlings representing 5–15 independent transgenic lines were used to present a visual average expression level (Figure 3). Although the camera assays are only semi-quantitative, captured light production from the transgenic seedlings clearly shows that CLE enhances luciferase activity in most tissues within the seedlings. Furthermore, an obvious correlation between CLE copy number and bioluminescence level can be seen in the images, further supporting the quantitative data derived from assaying leaf tissue (eg. pTDR#17(<) vs. pTDR#17-5(<<); pTDR#33(<) vs. pTDR#33-J(<<<<<) and pTDR#2(>) vs pTDR#2-L(>>>>>>>), Figure 3). Compared to the tissue distribution of luciferase activity in pTm35 and pTm35enh plants, CLE appears to enhance luciferase activity most effectively in leaves and least effectively in root tissues (Figure 3).

Mutational analysis of the CLE consensus sequence

The viral direct-repeat sequences, DR#37 and DR#40 (Table 1), each contain alterations (relative to the consensus CLE = GTGGTCCC) in one copy of the duplicated CLE elements (DR#37 = GTGGgCCC, and DR#40 = GTaGTCtC), yet retain the ability to enhance minimum CaMV 35S promoter function (Velten, *et al.* 2005). To further explore the effect of changes in CLE base composition, the CLE consensus ($G_1T_2G_3G_4T_5C_6C_7C_8$) was altered by systematically substituting adenosine for each of the 8 nucleotide positions. The resulting series of constructs ($pCLEm_{1–8}$) are each identical to the parent vector (pTCLE), except at a single position within each CLE copy within the direct repeat (Figure 4). Binary constructs harboring pTCLE, pTCLEm(1–8) and pTDR#33 were independently infiltrated into tobacco leaf tissues and assayed using the

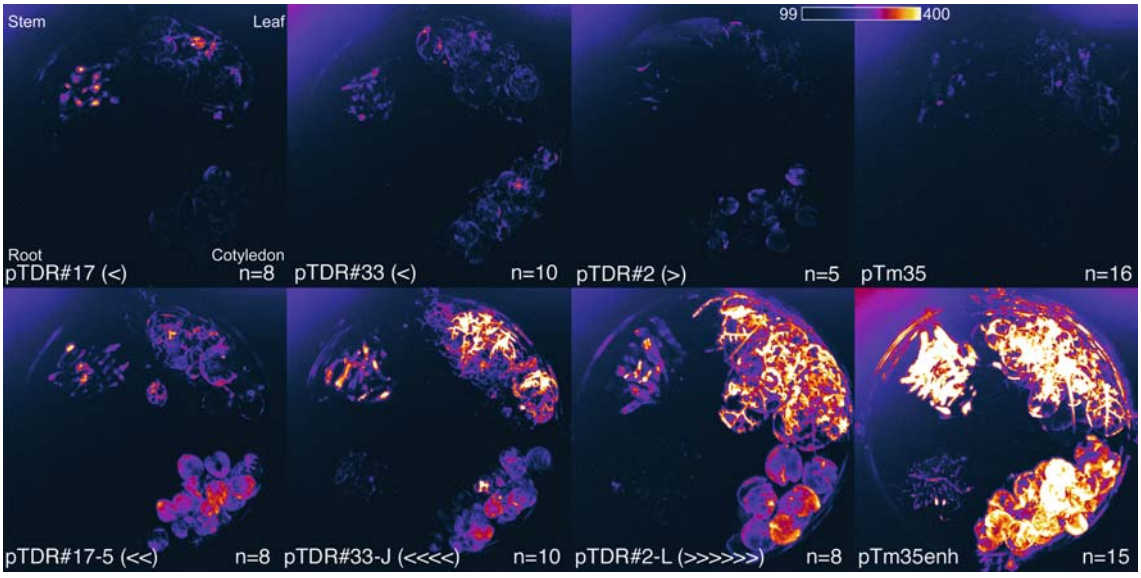

Figure 3. Bioluminescence assays showing tissue specific expression of the CLE motif. Herbicide resistant R_1 transgenic seedlings transformed with the indicated pT* test construct were dissected into leaves (upper right), cotyledons (lower right), roots (lower left) and stems (upper left) and floated on luciferin assay media. Captured images were enhanced and overlayed using NIH Image J software (v 1.32) {threshold values were maintained at lowest = 99 (black) and highest = 400 (white)}. The *n*-value shown indicates the number of independent transgenic lines (6–8 luciferase-positive seedlings from each line) assayed for each test construct.

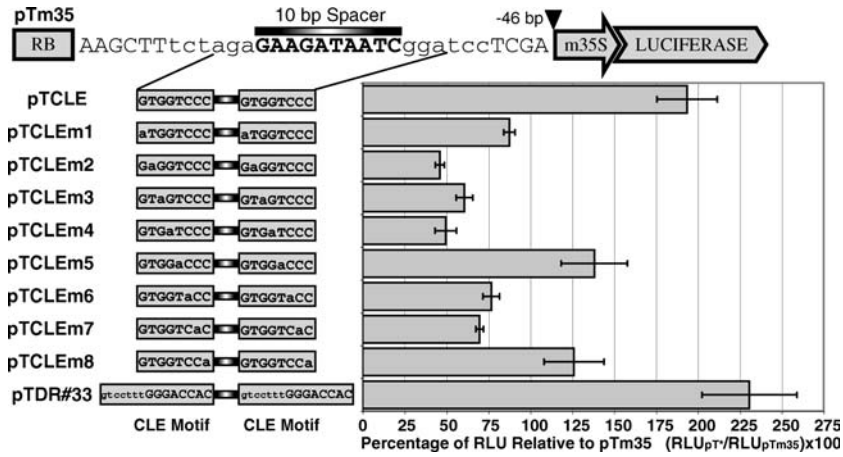

Figure 4. Mutational analysis of the CLE *cis*-acting sequence. Agrobacteria harboring pE1778-SUPER-RiLUC and the indicated test (pT*) binary vector were infiltrated into tobacco leaf tissues and 6–8 discs assayed for firefly and sea pansy luciferase activity using both *in vivo* and *in vitro* assays. Firefly luciferase activity from the pTCLEm* constructs (containing mutant CLE repeat) is reported relative to the activity from control infusions done using the minimal promoter::FiLUC plasmid (pTm35) ($[RLU_{pT*}/RLU_{pTm35}] \times 100$). Presented data are the mean values from all time points and both *in vivo* and *in vitro* assays. Error bars represent standard error. All tested elements (except pTDR#33) were significantly different from pTCLE at >95% confidence ($P < 0.05$, Student's *t*-test applied to raw data).

in vivo luciferase bioassay. Enhanced luciferase activity (~1.9- to 2.3-fold) for pTCLE and pTDR#33 was observed relative to the minimum CaMV 35S parent construct (pTm35) which agrees with our previous analysis (compare Figure 4 with Figure 2 and DR values listed in Table 1). The very similar activity enhancement observed with the pTCLE and pTDR#33 constructs indicates that neither the sequence flanking the CLE core consensus (gtcattt in pTDR#33), nor the spacing of the separation of repeats (pTCLE = 10 bp, pTDR#33 = 17 bp), appear to have a dramatic effect on CLE enhancer function.

Base substitution (Adenosine) at all positions ($G_1T_2G_3G_4T_5C_6C_7C_8$) reduced CLE-associated enhancement, with flanking bases (positions 1 and 8) and the central position 5 having the least impact. Mutations at positions 1, 2, 3, 4, 6, and 7 not only failed to enhance luciferase expression, but actually produced net reductions in activity, showing only 50–75% of the luciferase levels seen with the CLE-lacking minimum CaMV 35S parent construct (Figure 4, pTCLEm2, 3, 4, 6 and 7). The simplest explanation is that, at least in the case of the DNA cassettes tested, introduction of sequence upstream from the minimal 35S promoter appears to disrupt low level transcription associated with the minimum CaMV 35S promoter construct. In the case of wild type CLE (and to a lesser extent mutations m5 and m8) the loss of basal promoter function appears to be masked by CLE enhancer activity (missing in most of the mutant CLEs tested). The marked loss of enhancer function seen with the mutant CLE elements appears to require that both copies of the element be mutated since the CLE 'repeat' incorporated into the pTDR#40 test construct (Table 1) retained enhancer activity despite the second CLE copy containing two mismatches (GTaGTCtC) compared to the consensus sequence (Velten, *et al*. 2005).

Effects of the AC2 transactivating protein on CLE and non-CLE containing promoters

The AC2 (TrAP) protein from Pepper Huasteco Virus (PHV-AC2) was previously reported to enhance reporter gene activity (GUS) ~2- to 3-fold through interaction with a single or directly repeated CLE motif introduced upstream from a −46 to +8 bp minimal CaMV 35S-GUS promoter (Ruiz-Medrano, *et al*. 1999). To further examine the impact of PHV-AC2 on CLE function, the Agrobacterium transient assay system was used to examine the effect of PHV-AC2 co-expression on CLE/promoter-luciferase activity in both transient and stable expression systems. Promoter–luciferase fusions, containing two to twelve copies of the CLE element, were compared to constructs

harboring no CLE-like sequence components (pTm35, pTPGEL1). Transient co-expression of PHV-AC2 and luciferase was performed in both *N. tabacum* and *N. benthamiana* using Agrobacteria lines harboring pE1778-SUPER-AC2 (PHV-AC2 expression) co-infused with a second line carrying a test luciferase construct. The test constructs contain the firefly-intron gene driven by promoters containing: no CLE elements {Tm35 or TPGEL1}; 2 CLE elements {TDR#33(<) or TCLE(>)}; or 12 copies of CLE {TDR#2-L(>>>>>>)}. In the presence of co-expressed PHV-AC2, luciferase activity in both *Nicotiana* species was enhanced 2- to 3-fold relative to infusions where the pE1778-SUPER-AC2 construct was replaced with a negative control plasmid expressing GUS (pIG121) (Figure 5A). There was no correlation between the presence of CLE within the test promoter-luciferase construct and PHV-AC2 induced elevation in luciferase activity. The two test promoters that lack any CLE element (Tm35 and TPGEL1) were enhanced by co-expression of PHV-AC2 at least as effectively as the promoter harboring 12 copies of a virally derived CLE {pTDR#2-L(>>>>>>), Figure 5A}.

A similar promoter-independent elevation of reporter gene activity has been seen in infusions containing a mixture of Agrobacteria lines containing reporter gene constructs and promoter-gene fusions expressing viral suppressors of gene silencing. The reported suppressor-associated increases in transient gene expression are thought to result from suppressor-induced reduction in PTGS occurring within the infused regions (Schob *et al.*, 1997; Voinnet and Baulcombe, 1997; Anandalakshmi *et al.*, 2000; Llave *et al.*, 2000; Cazzonelli and Velten, 2003; Johansen and Carrington, 2001; Voinnet *et al.*, 2003; Vanitharani *et al.*, 2004). Viral TrAP protein homologs of PHV-AC2 have been shown to act as general suppressors of post-transcriptional gene silencing (PTGS) (Voinnet *et al.*, 1999; Van Wezel *et al.*, 2003; Selth *et al.*, 2004; Vanitharani *et al.*, 2004) and the observed PHV-AC2 mediated elevation of reporter gene expression in Agrobacteria infusions could result from suppression of silencing, as opposed to direct CLE/promoter activation.

As an alternative to examining PHV-AC2 function in the transient system, where PTGS is a proven factor, stably transformed *N. tabacum* plants (containing FiLUC constructs driven by promoters incorporating varying numbers of CLE) were also tested for PHV-AC2 activation. In these experiments one side of a transgenic tobacco leaf (separated by the midrib) was infiltrated with an Agrobacteria line containing pIG121 (negative GUS control, producing no viral suppressor) and the other half infused with either pE1778-SUPER-AC2 (PHV-AC2 expressing) or pBIN61-HcPro (producing the *Potato virus Y*; Helper component Protein suppressor of PTGS). The transgenic plants tested contained: no copy of CLE {pPZP2.5LuNt = PGEL1 promoter); one copy of CLE {pPZP35SLuNt = CaMV35S promoter, pTm35enh = enhanced minimal-35S promoter}; and 12 copies of CLE {pTDR#2-L (>>>>>>) = 12xCLE synthetic promoter}. Luciferase activities were measured and found to be enhanced by PHV-AC2 in all of the transgenic plant lines tested (2.1- to 2.7-fold), irrespective of the presence of CLE within the different luciferase promoters. In contrast, transient expression of the HcPro suppressor of PTGS produced little significant luciferase activation (20–50% increases) within the infused regions (Figure 5B). Activation of co-expressed luciferase reporter genes by PHV-AC2, in both transient and stable systems, does not correlate with the presence of CLE within test promoters and therefore, does not support direct interaction between the CLE motif and PHV-AC2 protein.

Discussion

The conserved late element (CLE) was originally identified by sequence analysis as a DNA sequence repeat present in many of the major dicot-geminiviral genomes (Arguello-Astorga *et al.*, 1994) and has subsequently been found to be markedly enriched within a comprehensive geminiviral sequence database (Velten *et al.*, 2005). CLE-like motifs have since been identified in several strong plant-functional promoter sequences (summarized in Table 1), and although not generally recognized as a *cis*-acting plant promoter element, is slightly enhanced within *Arabidopsis* promoter sequences (CLE = 3.9 in the -500 database vs. 2.79 for comparable random sequences) (Velten *et al.*, 2005). A sequence element (IIa; GTGGGCCCGT), identified within the rice PCNA promoter,

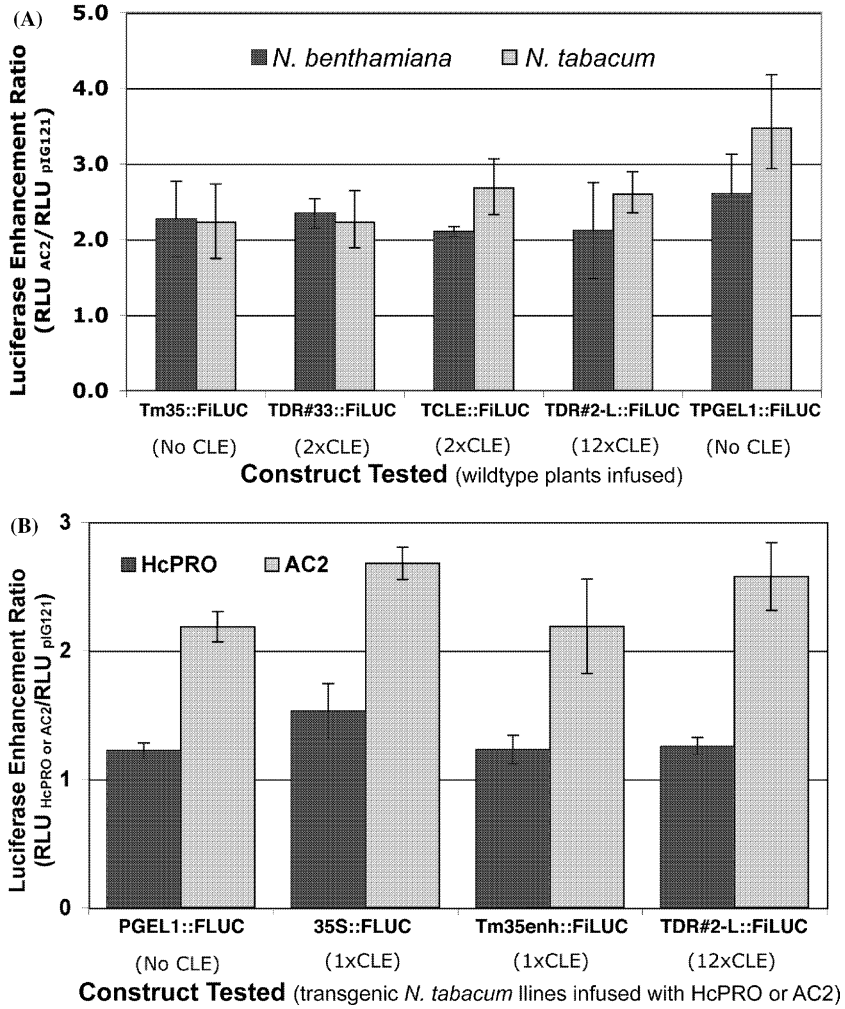

Figure 5. The effect of the PHV-AC2 protein on CLE function (transient and stable luciferase reporter expression). (A) Transient luciferase expression: Leaves from *N. tabacum* and *N. benthamiana* (as indicated) were infused with mixed Agrobacteria cultures containing equal densities of lines harboring the indicated promoter::luciferase test construct; and either pE1778-SUPER-AC2 (PHV-AC2 expressing) or pIG121 (negative, GUS-expressing control). Infusions were assayed for firefly luciferase activity using the *in vivo* assay. The luciferase enhancement ratio (RLU_{AC2}/RLU_{pIG121}) represents RLU emitted from the PHV-AC2 infusions divided by RLU from the pIG121 control. The value presented is the mean enhancement ratio from duplicate assays (error bars represent standard error). In both plant species reporter activity with AC2 was significantly different from the pIG121 controls at >98% confidence ($P < 0.02$, Student's t-test applied to raw data). (B) Stable luciferase transgenics: Transgenic *N. tabacum* expressing the indicated promoter::luciferase construct were infiltrated with an Agrobacteria line containing either pE1778-SUPER-AC2, pBIN61-HcPro or the pIG121 negative control. Leaf discs were assayed for luciferase activity using the *in vivo* assay. The luciferase enhancement ratio [(RLU_{HcPro} or AC2)/RLU_{pIG121}] is shown for both PHV-AC2 or HcPro infusions relative to the control (pIG121). The value presented is the mean enhancement ratio from duplicate assays (error bars represent standard error). Reporter activity enhancement with AC2 was different from pIG121 controls at >95% confidence ($P < 0.05$, Student's t-test applied to raw data), HcPRO enhancement was not statistically significant.

closely resembles the CLE consensus sequence and binds plant transcription factors (Kosugi *et al.*, 1995; Cubas *et al.*, 1999; Kosugi and Ohashi, 2002). The maize *GapC4* promoter also contains a *cis*-acting element (tccgGTGGGCCCgaaac) that closely resembles the CLE sequence and was shown to interact with components of tobacco leaf nuclear extracts (Geffers *et al.*, 2000). More recently it has been demonstrated, using a tobacco transient expression system, that directly repeated

copies of the CLE motif enhance reporter gene activity when placed upstream from a minimal CaMV 35S promoter (Velten *et al*., 2005). The finding that CLE; behaves as a positive enhancer of transcriptional regulation in plants, appears within elements that bind plant transcription factors, and is significantly enriched in the intergenic regions of viral genomes, strongly suggests that CLE, or closely related sequence elements, play some role in plant and viral gene regulation.

In order to better characterize the function of CLE in plant gene regulation, the impact of multiple copies of the CLE consensus on transient expression from a minimal CaMV 35S-luciferase gene fusion was examined in tobacco leaves infused with Agrobacteria. Increasing the number of CLE repeats elevated luciferase expression in an additive manner that was independent of the element's orientation (Figures 2 and 3). Constructs containing the greatest number of CLE motifs {pTDR#2-L(> > > > > >); 12xCLE and pTDR#33-J(> > > >); 8xCLE} were able to enhance luciferase activity in leaf and other tissues to levels just below those observed with a reconstructed CaMV 35S promoter that contains a single CaMV 35S enhancer domain (pTm35enh) (Figures 2 and 3). This finding is similar to previously reported enhancement of minimal promoter activity (−90 to +8, CaMV 35S) associated with multimerization (4x) of the G-box 10 base element (GCCACGTGCC) (Suzuki, *et al*., 1999). The enhancer like activity of the CLE motif was also analyzed in stably transformed tobacco tissues using whole-plant bioluminescence, with the finding that CLE element is able to enhance expression levels in most tissues present within the transgenic seedlings (Figure 3).

Mutational scanning analysis of the CLE element ($G_1T_2G_3G_4T_5C_6C_7C_8$) indicated that single nucleotide substitutions at positions 1, 2, 3, 4, 6 and 7 completely eliminates enhancer-like activity, while substitutions at 5 and 8 are less disruptive, only reducing relative enhancement (Figure 4). If, as the data suggests, the CLE element functionally tolerates at least some base variation at positions 5 and 8, the resulting consensus (GTGGNCCN) matches important promoter sequences identified in the rice PCNA (GTGGGCCCGT) and maize *GapC4* (GTGGGCCCG) promoters. The CLE element appears to be a possible target for plant encoded TCP domain transcription factors, consistent with reports of a biased frequency of occurrence for a CLE-like "TCP domain" binding consensus sequence (Gt/cGGNCCC) within *Arabidopsis* promoters (Kosugi and Ohashi, 2002). It is possible that TCP domain-containing transcription factors contribute to the observed CLE enhancer activity in tobacco since *Arabidopsis* promoters containing the TCP domain consensus binding element were found to function in transgenic tobacco and to show reduced activity after mutation of the element's core sequence (GGNCCC) (Kosugi and Ohashi 2002).

The exact function that the CLE element plays in viral gene regulation is less clear. The CLE motif was found to be enriched in regulatory regions such as the coat and movement protein promoters from the geminiviridae family (Arguello-Astorga *et al*., 1994). The presence of CLE in these intergenic regions correlates well with a noted conservation in amino acid sequence of the putative DNA-binding domain of the transactivator AC2 protein (TrAP; also known as AL2 or C2 protein) and it was suggested that CLE might be a functional target of AC2 (Arguello-Astorga *et al*., 1994; Sunter and Bisaro, 1992). It was experimentally shown, using a transient assay system (particle bombardment of tobacco leaf tissues), that the Pepper Huasteco Virus AC2 transactivating protein could enhance reporter gene activity of a construct containing 1 or more copies of the CLE motif fused upstream from a minimal CaMV 35S-GUS fusions (Ruiz-Medrano *et al*., 1999). However, both transient and stable transgene expression data (this paper) indicate that the PHV AC2 protein is capable of enhancing transcription of all tested promoter–reporter gene constructs in a manner independent of the presence of CLE-like elements within the test promoters (Figure 5). Hung and co-orkers (2001) also demonstrated, using a transient assay system (electroporation of tobacco protoplasts), that the *beangolden mosaic virus* (BGMV) late gene promoters (which lack CLE elements) were activated by the a PHV-AC2 homolog, the BGMV AL2 protein (Hung and Petty 2001). Recent demonstration that transient expression systems are subject to post-transcriptional gene silencing (Voinnet *et al*., 1999; Johansen and Carrington) and that viral encoded suppressors of gene silencing, such as HcPro, p19 and AC2 (from Tomato Leaf Curl, Tomato Yellow Leaf Curl and African

Cassava Mosaic viruses) can suppress PTGS silencing effects (Voinnet *et al*., 1999; Vanitharani *et al*., 2004), provide an alternative mechanism for the elevation of luciferase activity observed during co-expression of PHV-AC2 (Figure 5). It is possible that the enhanced reporter gene activity in transient systems results from PHV-AC2's impact on PTGS and not through a direct interaction between PHV-AC2 and CLE. However, it has also been shown that there appear to be differential roles for AC2-like proteins in mediating viral synergism and suppression of PTGS, implying multiple functions for the TrAP family of geminiviral proteins (Hao *et al*., 2003; Wang *et al*., 2003; Vanitharani *et al*., 2004). At present there remains some controversy as to whether AC2/AL2 (TrAP) proteins, specifically PHV-AC2, interact directly with the CLE element to affect promoter function.

The results presented here on the impact of PHV-AC2 expression in leaves of stable FiLUC transgenics raise additional questions regarding the role of PHV-AC2 as a possible suppressor of PTGS. In all transgenic lines tested, PHV-AC2 expression elevated the luciferase reporter gene activity (relative to Agrobacteria infusions containing the non-suppressing GUS control plasmid, pIG121, Figure 5b) by a factor of 2–3, while expression of a confirmed suppressor of PTGS, HcPro, had little, if any, significant effect (20–50% increase). The failure of HcPro to impact transgene expression in stable transformants is in contrast to what is routinely observed in Agrobacteria leaf infusion assays. During co-expression of PHV-AC2 or HcPro with various FiLUC constructs (including pTm35) in Agrobacteria-infused tobacco leaves, HcPro consistently elevates FiLUC activity by 4- to 8-fold while PHV-AC2 enhancement remains the same factor of $\sim$2 seen in the stable FiLUC transgenics (data not shown). The HcPro data in stable transgenics does not directly support the conclusion that significant PTGS is occurring in the transformed lines tested. Thus, if PHV-AC2 is actually functioning as a suppressor of silencing it appears to make use of different mechanisms then those employed by HcPro. Further analysis of the impact of PHV-AC2 expression on FiLUC transcription and silencing in both stable and transient systems, including characterization of mRNA levels and short RNAs, will be necessary to resolve the molecular mechanism(s) of PHV-AC2 function.

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