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Identification of *cis*-elements for ethylene and circadian regulation of the *Solanum melongena* gene encoding cysteine proteinase★

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Abstract

We have previously shown that the expression of *SmCP* which encodes *Solanum melongena* cysteine proteinase is ethylene-inducible and is under circadian control. To understand the regulation of *SmCP*, a 1.34-kb *SmCP* 5′-flanking region and its deletion derivatives were analyzed for *cis*-elements using *GUS* and *luc* fusions and by *in vitro* binding assays. Analysis of transgenic tobacco transformed with *SmCP* promoter-*GUS* constructs confirmed that the promoter region −415/+54 containing Ethylene Responsive Element ERE(−355/−348) conferred threefold ethylene-induction of GUS expression, while −827/+54 which also contains ERE(−683/−676), produced fivefold induction. Using gel mobility shift assays, we demonstrated that each ERE binds nuclear proteins from both ethephon-treated and untreated 5-week-old seedlings, suggesting that different transcriptions factors bind each ERE under varying physiological conditions. Binding was also observed in extracts from senescent, but not young, fruits. The variation in binding at the EREs in fruits and seedlings imply that organ-specific factors may participate in binding. Analysis of transgenic tobacco expressing various *SmCP* promoter-*luc* constructs containing wild-type or mutant Evening Elements (EEs) confirmed that both conserved EEs at −795/−787 and −785/−777 are important in circadian control. We confirmed the binding of total nuclear proteins to EEs in gel mobility shift assays and in DNase I footprinting. Our results suggest that multiple proteins bind the EEs which are conserved in plants other than *Arabidopsis* and that functional EEs and EREs are present in the 5′-flanking region of a gene encoding cysteine proteinase.

Introduction

Circadian rhythms are controlled by the 24-h clock and occur in prokaryotes and eukaryotes (Barak *et al.*, 2000). In plants, biological processes such as chloroplast movement, stomatal opening, leaf movements, and hypocotyl elongation are under circadian regulation (reviewed in Barak *et al.*, 2000). The core mechanism in circadian biology of all organisms is composed of a negative feedback loop, with positive and negative transcriptional regulators (Dunlap, 1999). Recent studies on *Arabidopsis* circadian rhythms have identified TIMING OF CAB EXPRESSION 1 (TOC1), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) as the main components of the core oscillator (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Strayer *et al.*, 2000;

★ EMBL/GenBank Data Library accession number(s) AF101032 (promoter of *S. melongena* cysteine proteinase gene, *SmCP*).

Matsushika *et al*., 2002). Microarray experiments suggest that 6% of *Arabidopsis* genes express mRNAs that peak at different times in the 24-h clock (Harmer *et al*., 2000). These genes can be further divided into six clusters based on peak expression time (Harmer *et al*., 2000).

Computational studies, deletion analysis and site-directed mutagenesis have identified a conserved EE motif [(AA)AA**T**ATCT] in the promoters of genes with peak expression in late light (Harmer *et al*., 2000; Xu and Johnson, 2001; Michael and McClung, 2002). The EE differs only by a single nucleotide (bolded) from the CCA1-binding site (AAAAA**A**ATCT), the morning element in promoters of genes showing peak expression in early light (Michael and McClung, 2002). In the *CATALASE 3* promoter, a **T** to **A** substitution in the EE converted it to a morning element resulting in peak expression in early light (Michael and McClung, 2002). Alabadí *et al*. (2001) showed that peak expression of *TOC1* occurs in late light, its promoter has a conserved EE and the overexpression of either LHY or CCA1 leads to a disruption of circadian regulation and a decline in *TOC1* mRNA, indicating that both CCA1 and LHY are negative regulators of *TOC1* expression. Although *E. coli*-expressed recombinant CCA1 and LHY has each been shown to bind the EE in the *TOC1* promoter (Alabadí *et al*., 2001), neither *in vitro* binding of total nuclear protein extracts to the EE nor DNase I footprints at the EE has been previously reported.

The G-box occurs in a broad range of plant promoters that are affected by environmental cues. It is also a light-regulated motif (Chattopadhyay *et al*., 1998; Xu and Johnson, 2001) and has the same consensus sequence as the E-box of animal gene promoters that functions as a circadian enhancer motif (Hao *et al*., 1997; Dunlap, 1999). Using *in vitro* binding studies, Martinez-Garcia *et al*. (2000) demonstrated that the transcription factor PIF3 specifically binds the G-box at promoters of light-regulated *Arabidopsis* genes like *CCA1*, *LHY*, *SPA1* and *RBCS-1A* and interacts with the light-activated form of phytochrome B.

Ethylene is an endogenous hormone regulating many plant processes from seed germination to plant senescence (Bleecker and Kende, 2000) and acts as a stress hormone during adverse biotic and abiotic conditions. The promoters of various genes that are ethylene-inducible contain EREs. An 8-nucleotide ERE (ATTTCAAA) in the carnation *GST1* promoter mediating senescence-related expression, was identified by DNase I footprinting (Itzhaki *et al*., 1994). It shows significant homology to the ERE (AAATTCAAA) of the tomato *E4* promoter, and lies within a larger region protected from DNase I digestion (Montgomery *et al*., 1993). The GCC box in promoters of defense-related genes also mediates ethylene-responsiveness (Ohme-Takagi and Shinshi 1990; Eyal *et al*., 1993), but has not been identified in promoters of genes associated with fruit ripening or petal senescence, suggesting that these *cis*-elements are distinct (Ohme-Takagi *et al*., 2000).

We have cloned and characterized *SmCP* which encodes a cysteine proteinase in *Solanum melongena* (Xu and Chye, 1999). The localization of *SmCP* mRNA by *in situ* hybridization revealed that its expression coincides with developmental events leading to programmed cell death in plant tissues, suggesting its role in protein degradation (Xu and Chye, 1999). Northern blot analysis has shown that its mRNA is ethylene-inducible and is under circadian control with peak expression in late light (Xu *et al*., 2003). We have previously observed that *SmCP* and *rbcS* differ in their peak expression times and had suggested that protein degradation and photosynthesis, which are catabolic and anabolic events, respectively, could be separated by circadian regulation in opposite phases to maximize their functions (Xu *et al*., 2003). Expression of tobacco cysteine proteinase *CYP-8* mRNA also peaks in late light (Linthorst *et al*., 1993), but the molecular basis for circadian control of any cysteine proteinase gene promoter has yet to be established. We have already shown by EMSA and DNase I footprinting that a G-box (CACGTG) is located at −85/−80 in the 5′-flanking region of *SmCP* (Xu *et al*., 2003). G-box binding activity was stronger in senescent than young fruits, and in circadian-regulated leaves, stronger binding activity coincided with peak circadian expression of *SmCP,* suggesting that G-box binding coincides with enhanced *SmCP* transcription (Xu *et al*., 2003). In order to further understand the regulation of *SmCP*, we identify here the *cis*-elements (EREs and EEs) in the *SmCP* promoter and show their importance in gene regulation. We present evidence in EMSA and in DNase I footprinting that total nuclear proteins bind the EEs.

Materials and methods

Plant material, growth conditions and ethephon treatment

Eggplant/brinjal (*S. melongena*) and tobacco (*Nicotiana tabacum* var Xanthi) were grown in a growth chamber at 24 °C under a light/dark regime of 12 h light (08:00–20:00)/12 h dark (20:00–08:00). Five-week-old *S. melongena* or transgenic tobacco seedlings were sprayed with ethephon following Greenberg and Ausubel (1993). Samples from treated and control *S. melongena* seedlings were harvested 24 h after spray for nuclear protein extraction. Northern blot analysis was also carried out using RNA samples from ethephon-treated and control seedlings, 24 h after treatment, to confirm ethephon-induction of *SmCP* expression in nuclear protein extracts before they were used in EMSAs.

Northern blot analysis

Total RNA was isolated from *S. melongena* by the method of Nagy *et al.* (1988) and northern blot analysis was carried out as previously described by Xu *et al.* (2003). Total RNA (20 μg) was denatured at 50 °C in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gel and blotted onto Hybond-N (Amersham) membrane. To ensure that equal amounts of RNA were used, RNA was stained with ethidium bromide after gel electrophoresis. The RNA blot was hybridized at 42 °C with [^{32}P]dCTP-labeled *SmCP* cDNA in a solution containing 50% deionized formamide, 1× Denhardt's solution, 6× SSPE, 0.1% SDS, 100 μg/ml denatured sonicated salmon sperm DNA and 10% dextran sulfate. The blot was washed at 65 °C in 0.1× SSC, 0.1% SDS. Bands were detected by autoradiography.

Construction of SmCP *promoter*-GUS *fusions*

Various deletions of the *SmCP* 5′-flanking region were PCR-amplified using different primer pairs (Figures 1 and 2A) and plasmid pSm8 DNA as template. Plasmid pSm8 is a pBluescript derivative that contains *SmCP* on a 5-kb *Eco*RI genomic DNA fragment (Xu *et al.*, 2003). Each 25-μl PCR reaction consisted of 50 ng pSm8 DNA, 10 pmol of each primer, 1U *Taq* polymerase (Perkin Elmer), 2.5 μl 10 × PCR buffer, 1.5 μl of 25 mM $MgCl_2$ and 0.5 μl each of 10 mM dNTPs. PCR was initiated with denaturation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 min, 45 °C

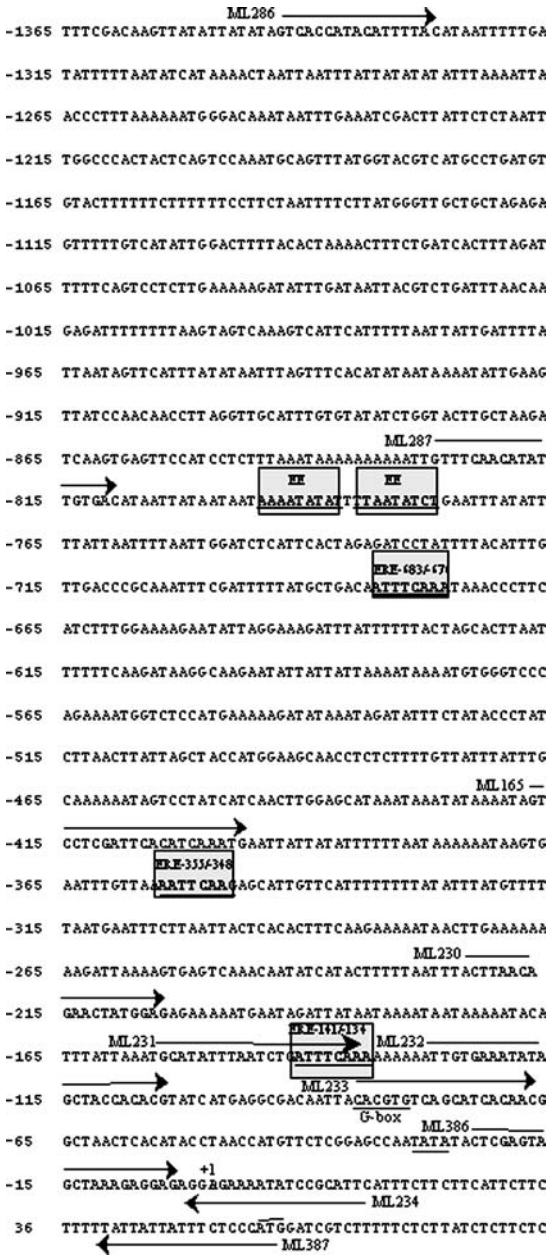

Figure 1. Nucleotide sequence of the *SmCP* 5′-flanking region. The putative EREs and EEs are marked in gray boxes. Arrows indicate the location of the primers on the *SmCP* 5′-flanking sequence. The TATA box and the G-box are underlined. The ATG start codon is overlined. The start site of transcription (nucleotide *A*) as mapped by primer extension analysis (Xu *et al.*, 2003) is marked +1.

for 30 s and 68 °C for 2 min, and extension at 72 °C for 10 min. The various PCR-generated fragments were cloned into vector pGEM-T Easy (Promega), in an orientation with the 5′-end of the *SmCP* promoter adjacent to the *Spe*I site on the polylinker of pGEM-T Easy. The PCR fragments in these pGEM-T Easy derivatives were then verified by DNA sequence analysis.

Subsequently, the various PCR-generated 5′-flanking sequences were fused to the *GUS* reporter gene in binary vector pBI101.3 (Clontech) which confers kanamycin-resistance (Jefferson *et al.,* 1987). To this end, plasmid pSm11 (Figure 2A) was first created by cloning a Klenow-treated 552-bp *Nco*I–*Nco*I (−498/+54) fragment from pSm8 into the *Sma*I site of pBI101.3. A unique *Xho*I site (−23/−18) on the 552-bp *SmCP* promoter fragment and an upstream *Xba*I site on the polylinker region of pSm11 was used in cloning eight other promoter fragments flanking *SmCP*. The *Spe*I-*Xho*I 5′-fragment of each deletion was subcloned from the pGEM-T Easy derivative into the *Xba*I-*Xho*I sites of pSm11. Altogether nine deletions of the 5′-flanking region were analyzed (Figure 2A); the largest fragment (1.34-kb) contains putative EREs and EEs fused upstream of *GUS* (Figure 2).

Construction of SmCP promoter-luciferase fusions

SmCP promoter-luciferase (*SmCP* promoter-*luc*) fusions were made using the 1.7-kb firefly luciferase reporter gene from pGEM-*luc* (Promega). The *Xho*I site in pGEM-*luc* was destroyed by *Xho*I digestion followed by filling-in with Klenow to create plasmid pSm124. A 78-bp *SmCP* promoter fragment (−23/+54), PCR-amplified using forward primer ML386 5′-AT<u>AAGCTT</u>***CTCGAG***<u>**TAGCTAAAGAGGAGA**</u>-3′ (*Hin*dIII site underlined; *SmCP* 5′-flanking sequence bolded with *Xho*I site in italics) and reverse primer ML387 5′-AT<u>GGATCC</u>**GGAGAAATAATAAT**-3′ (*Bam*HI site underlined) in the pGEM-T Easy derivative pSm125, was cloned into the *Hin*dIII and *Bam*HI sites of pSm124 to create pSm126. To generate various *SmCP* promoter-*luc* fusions in pBI101.3, the 1.8-kb *Xho*I-*Sac*I *GUS* fragment from each *SmCP* promoter-*GUS* construct (*Sac*I site at 3′-end of *GUS*) was replaced by the 1.7-kb *Xho*I-*Sac*I *luc* fragment from the *SmCP* promoter-*luc* plasmid pSm126.

To generate constructs with mutant EEs on the −827/+54 *SmCP* promoter fragment, three 0.8-kb PCR-amplified fragments were generated using template pSm8, reverse primer ML234, and either

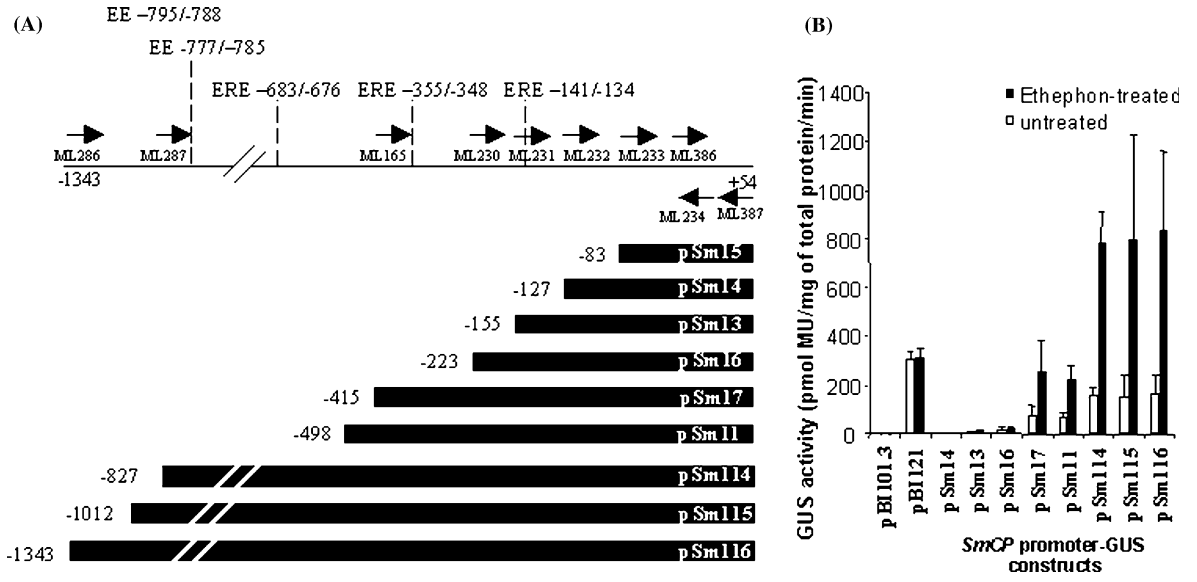

Figure 2. Deletions generated in construction of *SmCP* promoter-*GUS* fusions for analysis of putative EREs. (A) Schematic representation of the *SmCP* 5′-end deletions indicated by black bars (not to the scale) with respect to the putative EEs and EREs. PCR primers used to generate these deletions are marked (arrows). (B) GUS activities of tobacco lines transgenic for *SmCP* promoter-*GUS* constructs. Plants were sprayed with ethephon, incubated for 24 h and assayed for GUS activity. Error bars indicate standard error of mean. Data represent the average of at least three independent tobacco transformants from the same construct.

one of forward primers ML429 5′-GTTTCAACA TATTGTGACATAATTATAATAAT**CCCCGCG CG**TTTAATATCTG-3′ (mutant EE −795 to −787 in bold), ML430 5′-GTTTCAACAT ATTGTGACATAATTATAATAATAAAATA TATT**GGCCGCGTG**GAATTTATATTT-3′ (mutant EE −785 to −777 in bold) or ML431 5′-GTTTCAACATATTGTGACATAATTATAA TAAT**CCCCGCG**ATT**GGCCGCGCG**GAATTTA TATTT-3′ (mutant EEs −795 to −787 and −785 to −777 in bold). Each fragment was cloned into pGEM-T Easy in an orientation with the 5′-end of the *SmCP* promoter adjacent to the *Spe*I site on the polylinker of pGEM-T Easy. The PCR fragments in these pGEM-T Easy derivatives were then verified by DNA sequence analysis. Each *Spe*I-*Xho*I fragment of the *SmCP* promoter (−827/ +54) was subcloned from the pGEM-T Easy derivative into the *Xba*I-*Xho*I sites of pSm130 to generate pSm140, pSm141 and pSm142.

Generation of transgenic plants using Agrobacterium*-mediated transformation*

Constructs of the *SmCP* promoter fused to either *GUS* or *luc* were mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating. Transgenic tobacco plants were generated by *Agrobacterium*-mediated transformation of leaf discs. Leaf explants (0.5 × 0.5 cm) from wild-type tobacco grown *in vitro* were soaked in *Agrobacterium* solution ($OD_{600} = 0.1$ to 0.3, using UV-spectrophotometer Shimadzu Model UV-1206) for about 10 min and transferred to plant regeneration medium containing MS basal (MSO, Murashige and Skoog, 1962) agar media (pH 5.8) supplemented with 6-benzylaminopurine (1 mg/ml) and α-naphthalene-acetic acid (NAA) at 0.2 mg/ml final concentration. After co-cultivation for 2 days, the explants were washed in liquid MSO supplemented with carbenicillin (500 mg/l) for over 2 h to remove *Agrobacterium* and transferred to plant regeneration medium supplemented with kanamycin (100 mg/l) and carbenicillin (500 mg/l) for callus and shoot regeneration. After 3 weeks, shoots were subcultured onto MSO supplemented with NAA (0.1 mg/ml), kanamycin (200 mg/l) and carbenicillin (500 mg/l) for root regeneration. In ethylene-induction assays, transgenic tobacco transformed with plasmids pBI121 and pBI101.3 were used as positive and negative controls, respectively.

Seed sterilization and germination

Transgenic tobacco seeds were sterilized in 70% ethanol for 1 min followed by 10 min in 20% bleach (Chlorox) and washed thoroughly several times with sterilized water. After sterilization, seeds were germinated on MSO agar (0.8%) supplemented with kanamycin (200 mg/l), and transferred to soil in a growth chamber after 2 weeks. Control wild-type seeds were also sterilized the same way and germinated on MSO agar without antibiotic selection.

GUS assays

Leaf extracts from untreated and ethephon-treated seedling tissue, collected 24 h after treatment were analyzed for GUS activity by fluorometric quantification of 4-methylumbelliferone (MUG) using substrate β-D-glucuronide as described by Yang *et al.* (2000). Total protein concentration of the tissue homogenate was determined according to Bradford (1976) with the Protein Assay Kit I (BioRad). GUS activity was expressed as pmol of product generated per mg of total protein per min.

Luciferase assays

Replicates of leaves from individual transgenic lines were harvested in 1.5-ml eppendorf tubes, frozen in liquid nitrogen and stored at −80 °C until further use. The frozen tissue was ground to powder and resuspended at room temperature in luciferase extraction buffer containing 100 mM potassium phosphate buffer (pH 7.5) and 1 mM dithiothreitol (DTT), followed by centrifugation for 5 min at 4 °C as described by Ow *et al.* (1986). The supernatant was retained for measurement of luminescence using a microtiter plate-reader (POLARstar from BMG Technologies, USA). Measurements were read for 10 s with an initial 4 s delay. Tissue homogenate (40 μl) was added in three replicates to a 96-well microtiter plate (Nunc Cat. No. 236105), and diluted with 60 μl of luciferase extraction buffer before placing in the luminometer. Luciferase assay reagent (100 μl) from Promega (Cat. No. 1511) was added to each well and read after a 4 s delay.

Preparation of nuclear proteins

Nuclear proteins were prepared following Martino-Catt and Kay (1994) from 5-week-old seedlings, grown in growth chambers at 24 °C with a 12 h light (08:00–20:00)/12 h dark (20:00–08:00) cycle. For binding studies involving EEs, samples were collected at peak (17:00) and low (06:00) circadian expression times as previously established by Xu *et al.* (2003). For binding studies involving EREs, samples from 5-week-old seedlings (ethephon-treated or untreated) were used.

S. melongena fruits are purple when young and on ripening changes colour to yellow at about 50 days after pollination (DAP) (Xu and Chye, 1999). Correspondingly, *SmCP* mRNA expression in fruits increases when the fruits turn yellow (Xu and Chye, 1999). We have previously used small purple fruits harvested 20 DAP as "young" fruit and yellow fruit harvested 60 DAP as "senescent" fruit (Xu and Chye, 1999). Here, for preparation of nuclear extracts for binding studies involving EREs, samples were harvested from small young (purple) fruits and senescent (yellow) fruits. Following protein determination (Bradford, 1976), aliquots of nuclear proteins were frozen in liquid nitrogen and stored at −80 °C until further use.

Electrophoretic mobility shift assays (EMSAs)

For binding studies to the EREs, two pairs of 40-mers ML312/ML313 (Figure 3A) and ML304/ML305 (Figure 4A) corresponding to the two putative EREs at −355/−348 and -683/−676 respectively, were synthesized, annealed and end-labeled with ^{32}P-dCTP by filling-in with Klenow. To investigate the specificity of the conserved sequence in binding, correspondingly pairs of ERE mutant 40-mers ML314/ML315 (Figure 3A) and ML306/ML307 (Figure 4A) were used in EMSA. The EEs(−795/−777) were investigated using ^{32}P-dCTP-labeled probes consisting of annealed 40-mers ML425/ML426 containing the two putative EEs(−795/−787 and −785/−777) and the mutant probe ML427/ML428 for EE(−795/−787)-mut (Figure 6A). Similarly, EMSAs on the EE(−785/−777) were investigated using ^{32}P-dCTP-labeled probes consisting of annealed mutant probe ML581/ML582 and wild-type probe ML425/426 (Figure 6A). Unlabeled oligonucleotides were used as competitors in binding.

The ^{32}P-dCTP-labeled mutant probes were mut1(−795/−792) ML587/ML588, mut2(−791/−788) ML589/ML590, mut3(−786/−784) ML591/ML592, mut4(−784/−781) ML585/ML586 and mut5(−780/−777) ML583/ML584 (Figure 7A). Binding reactions were carried out as according to Xu *et al.* (2003) and analyzed by running the reaction mix on a 6% non-denaturing polyacrylamide gel, followed by drying and autoradiography.

DNase I footprinting at EEs

The coding strand probe was prepared by linearizing pGEM-T Easy (Promega) derivative pSm117 containing the *SmCP* promoter region (−827/−706) with *Nco*I and end-labeling with ^{32}P-dCTP using Klenow. After removal of unincorporated ^{32}P-dCTP using Microspin G-25 columns (Amersham Pharmacia Biotech.), the ^{32}P-labeled probe was released from the labeled linearized plasmid by *Spe*I digestion. The probe was purified using a preparative non-denaturing 5% polyacrylamide gel. DNase I footprinting reactions were carried out as described previously (Xu *et al.*, 2003), and Maxam-Gilbert sequencing (G + A) reactions of the labeled promoter fragments were performed according to Sambrook *et al.* (1989). Samples were analyzed on an 8% polyacrylamide sequencing gel followed by autoradiography of the dried gel.

Results

Identification of putative cis*-elements in the* SmCP *5′-flanking region*

Our previous analysis of the *SmCP* 5′-flanking sequence had revealed the presence of a highly conserved 8-nucleotide ERE(−141/−134) (Xu *et al.*, 2003) which is identical to the ERE (ATTTCAAA) of carnation (*GST1*), a gene associated with petal senescence (Itzhaki *et al.*, 1994). However, this putative ERE in *SmCP* was not protected from DNase I (Xu *et al.*, 2003). Hence further analysis of a larger 1.34-kb 5′-flanking region (Figure 1) was deemed necessary to locate other possible EREs and EEs to elucidate the regulation of *SmCP* expression. A second putative ERE (ATTTCAAA) identical to that in *GST1* was observed at −683/−676, while a third

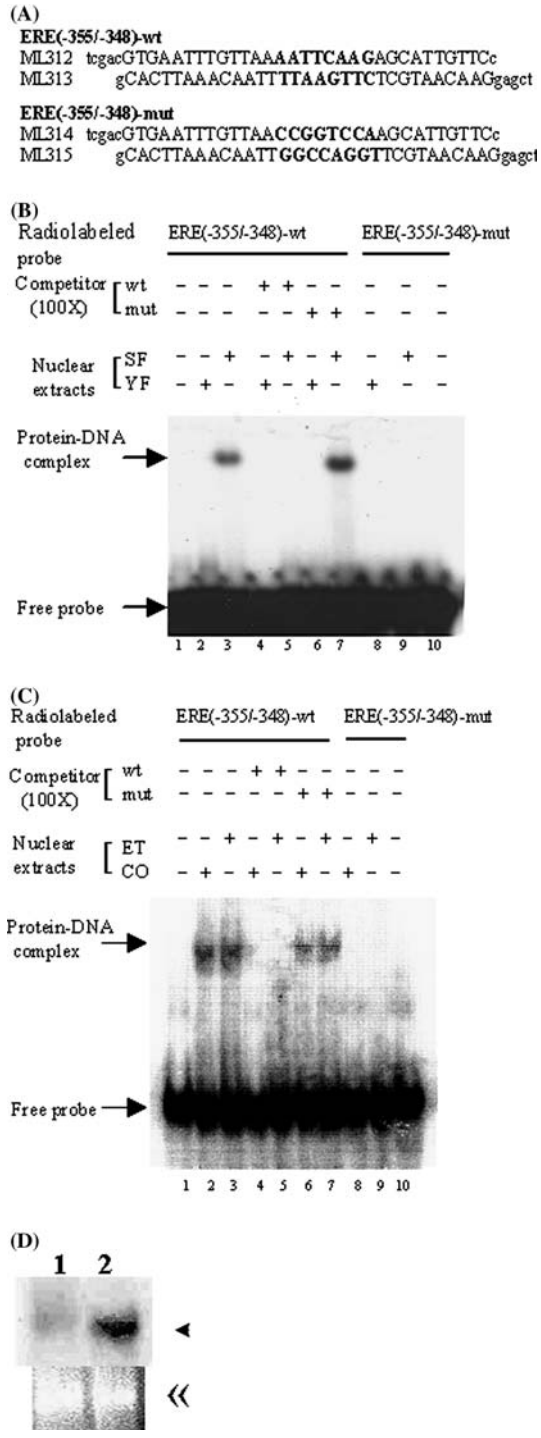

Figure 3. EMSAs on the ERE(−355/−348) in the *SmCP* promoter region. (A) Nucleotide sequences of double-stranded oligonucleotides in EMSAs. The mutated nucleotides in ERE(−355/−348)-mut and their corresponding sequences in ERE(−355/−348)-wt are shown in bold. Upper-case letters correspond to the region of the *SmCP* promoter with the putative ERE. Lower-case letters represent the additional sequences designed for end-labeling. (B) Interaction of nuclear proteins from *S. melongena* seedlings with ERE(−355/−348)-wt and ERE(−355/−348)-mut probes. Crude nuclear protein (10 μg) from senescent fruits (SF in lanes 3, 5, 7, 9) or young fruits (YF in lanes 2, 4, 6, 8) was incubated with end-labeled ERE(−355/−348)-wt (lanes 1–7) or ERE(−355/−348)-mut (lanes 8–10), in the absence (lanes 2, 3, 8 and 9) or presence of a hundredfold molar excess of unlabeled competitor, ERE(−355/−348)-wt (lanes 4 and 5) or ERE(−355/−348)-mut (lanes 6 and 7). Lanes 1 and 10 are free probes without addition of crude nuclear proteins. (C) Interaction of nuclear proteins from *S. melongena* seedlings with ERE(−355/−348)-wt and ERE(−355/−348)-mut probes. Crude nuclear protein (10 μg) from 5-week-old seedlings, treated with ethephon (ET in lanes 2, 4, 6, 8) or untreated (CO in lanes 3, 5, 7, 9), was incubated with end-labeled ERE(−355/348)-wt (lanes 1–7) or ERE(−355/−348)-mut (lanes 8–10), in the absence (lanes 2, 3, 8 and 9) or presence of a hundredfold molar excess of unlabeled competitor, ERE(−355/−348)-wt (lanes 4 and 5) or ERE(−355/−348)-mut (lanes 6 and 7). Lanes 1 and 10 are free probes without addition of crude nuclear proteins. (D) Northern blot analysis on the leaf samples harvested from 5-week-old *S. melongena* seedlings, used for nuclear protein extractions. The samples were harvested 24 h after the ethephon treatment. Control seedlings (lane 1) and ethephon-treated (lane 2). The northern blot with 20 μg total RNA per lane was hybridized to ^{32}P-labeled *SmCP* cDNA. Black arrowhead indicate the 1.5 kb *SmCP* mRNA and double arrowheads indicate 18S rRNA.

(AATTCAAG) with two-nucleotide mismatches (underlined) was detected at −355/−348 (Figure 1). The 1.34-kb 5′-flanking region also contains putative EEs [(AA)AATATAT] at −795/−787 with one nucleotide mismatch (underlined) to the consensus 9-nucleotide motif (AAAATATCT; Harmer *et al*., 2000) and another at −785/−777 (TTAATATCT) which retains only the 7-nucleotide core (AATATCT; Xu and Johnson, 2001).

Analysis and identification of EREs in the SmCP *5′-flanking region*

To test the functional relevance of these putative elements in transcriptional regulation of *SmCP* expression, a series of 5′-deletions progressively lacking the predicted elements were generated and fused upstream of the *GUS* reporter gene (Figure 2A). These *SmCP* promoter-*GUS* fusion genes were used in *Agrobacterium*-mediated transformation of tobacco. The transgenic lines thus obtained had different transgenic expression levels within the same constructs, depending upon the

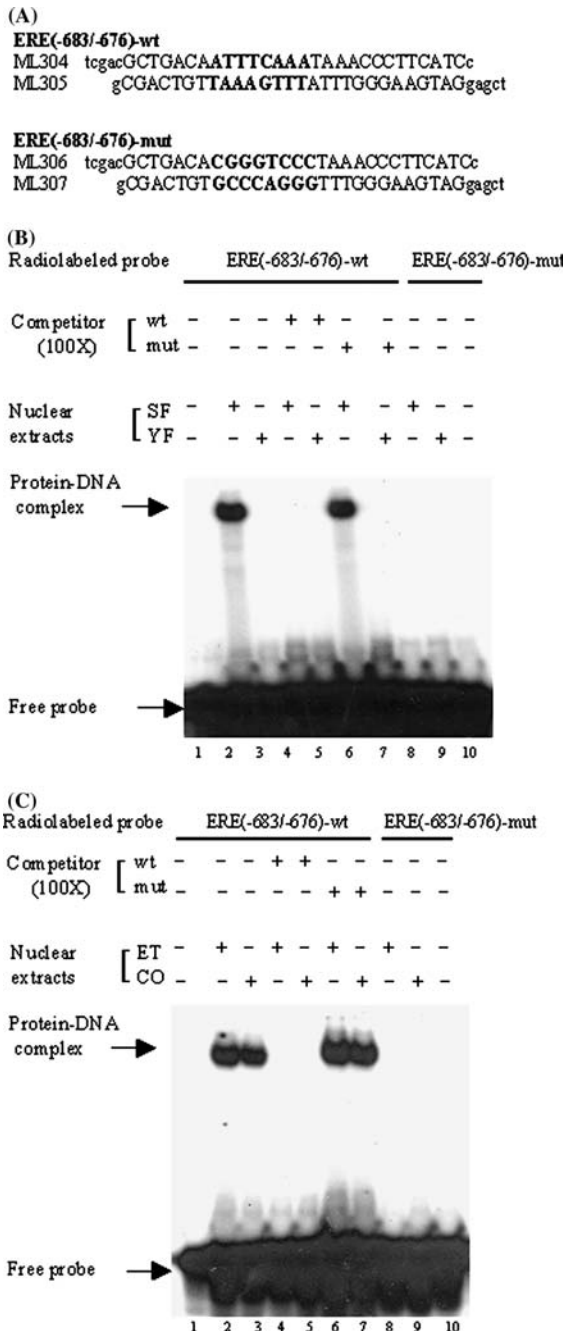

Figure 4. EMSAs on the ERE(−683/−676) in the *SmCP* promoter region. (A) Nucleotide sequences of double-stranded oligonucleotides in EMSAs. The mutated nucleotides in ERE(−683/−676)-mut and their corresponding sequences in ERE(−683/−676)-wt are shown in bold. Upper-case letters correspond to the region of the *SmCP* promoter with the putative ERE. Lower-case letters represent the additional sequences designed for end-labeling. (B) Interaction of nuclear proteins from *S. melongena* seedlings with ERE(−683/−676)-wt and ERE(−683/−676)-mut probes. Crude nuclear protein (10 μg) from senescent fruits (SF lanes 2, 4, 6, 8) or young fruits (YF in lanes 3, 5, 7, 9) was incubated with end-labeled ERE(−683−676)-wt (lanes 1–7) or ERE(−683/−676)-mut (lanes 8–10), in the absence (lanes 2, 3, 8 and 9) or presence of a hundredfold molar excess of unlabeled competitor, ERE(−683/−676)-wt (lanes 4 and 5) or ERE(−683/−676)-mut (lanes 6 and 7). Lanes 1 and 10 are free probes without addition of crude nuclear proteins. (C) Interaction of nuclear proteins from *S. melongena* seedlings with ERE(−683/−676)-wt and ERE(−683/−676)-mut probes. Crude nuclear protein (10 μg) from five-week-old seedlings, treated with ethephon (ET in lanes 2, 4, 6, 8) or untreated (CO in lanes 3, 5, 7, 9), was incubated with end-labeled ERE(−683−676)-wt (lanes 1–7) or ERE(−683/−676)-mut (lanes 8–10), in the absence (lanes 2, 3, 8 and 9) or presence of a hundredfold molar excess of unlabeled competitor, ERE(−683/−676)-wt (lanes 4 and 5) or ERE(−683/−676)-mut (lanes 6 and 7). Lanes 1 and 10 are free probes without addition of crude nuclear proteins.

integration of the T-DNA. In order to keep the fluctuations in the expression levels of independent transformants and standard error of mean low, we analyzed three to five independent transformants expressing maximum GUS activity from all promoter-*GUS* constructs except pSm15 (which did not show any activity). The controls used in the assay were transformants of pBI101.3 (promoterless-*GUS*) and pBI121 (CaMV35S-*GUS*). As shown in Figure 2B, the GUS activities of both ethephon-treated and untreated tissues were of the same low levels in lines transformed with pSm14 (−127/+54), pSm13 (−155/+54) and pSm16 (−233/+54). Both pSm13 and pSm16 contain putative ERE(−141/−134) which lacked protection in DNase I footprinting (Xu *et al*., 2003). EMSA studies also revealed that oligos corresponding to ERE(−141/−134) did not bind nuclear proteins extracted from ethephon-treated or untreated leaves, senescent or young fruits (data not shown). Upon ethephon treatment, there were about threefold increases of GUS activities in tobacco lines transformed with pSm17 (−415/+54) and pSm11 (−498/+54) containing ERE(−355/−348) while about fivefold increases were seen in transformants of pSm114 (−827/+54), pSm115 (−1012/+54) and pSm116 (−1343/+54) containing additional ERE(−683/−676).

Nuclear proteins interact with ERE(−355/−348)

To confirm the function of ERE(−355/−348), EMSAs was carried out using nuclear proteins

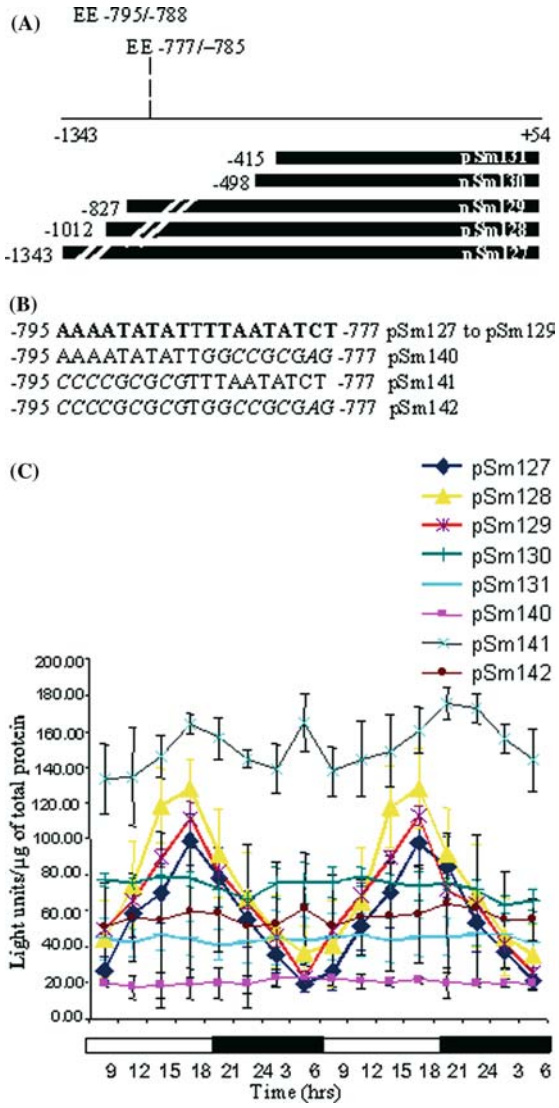

Figure 5. Effect of various *SmCP* 5′-end deletions and mutations in the EEs on circadian regulation of *luc* expression. (A) Schematic representation of the various constructs of *SmCP* promoter-*luc*. The black bars (not to the scale) represent different deletions in the 5′-flanking sequence of *SmCP*. The positions of the putative EEs are marked. (B) Nucleotide sequence of the *SmCP* promoter region between −795/−777 containing two EEs (bolded) and their corresponding mutations with nucleotide changes shown in italics. (C) Luciferase activity of tobacco lines transformed with various *SmCP* promoter-*luc* constructs pSm127 to pSm131 and mutant *SmCP* promoter-*luc* constructs pSm140 to pSm142. Samples were collected from 5-week-old tobacco seedlings grown at 24 °C under 12 h light/12 h dark period. Traces present average values ($n = 3$) from individual independent transgenic lines and error bars indicate standard error of mean. The bars beneath the graph indicate the light and dark regime, with subjective day indicated by white bars and subjective night indicated by black bars.

from senescent fruits (enriched with endogenous ethylene) or young fruits and annealed primer pair ML312/ML313 or its corresponding mutant ML314/315 (Figure 3A). The ERE(−355/−348)-wt probe showed binding to nuclear proteins from senescent (Figure 3B, lane 3) but not young fruits (Figure 3B, lane 2). In contrast, the corresponding ERE(−355/−348)-mut probe did not bind both senescent (Figure 3B, lane 9) and young fruit extracts (Figure 3B, lane 8). Addition of a hundredfold excess of unlabeled ERE(−355/−348)-wt competed and prevented the binding of labeled ERE(−355/−348)-wt to nuclear proteins from senescent fruits (Figure 3B, lane 5). The sequence-specific binding of the senescent fruit extract was further demonstrated when unlabeled ERE(−355/−348)-mut, could not compete out labeled ERE(−355/−348)-wt in binding (Figure 3B, lane 7).

The ERE(−355/−348) also showed binding to nuclear proteins from both untreated (Figure 3C, lane 2) and ethephon-treated *S. melongena* seedlings (Figure 3C, lane 3). Binding was competed by a hundredfold excess of unlabeled ERE(−355/−348)-wt, in both untreated (Figure 3C, lane 4) and ethephon-treated samples (Figure 3C, lane 5). In contrast, the corresponding ERE(−355/−348)-mut, showed no binding to untreated (Figure 3C, lane 8) and ethephon-treated samples (Figure 3C, lane 9). Unlabeled ERE(−355/−348)-mut could not compete against labeled ERE(−355/−348)-wt in binding (Figures 3C, lanes 6–7), confirming binding specificity of the latter.

The ethephon-induction of *SmCP* in the tissues harvested for nuclear protein extracts was investigated by northern blot analysis on total RNA extracted from both ethephon-treated and control seedlings. The results show an induced *SmCP* expression in the ethephon-treated sample in comparison to the control (Figure 3D).

Nuclear proteins interact with ERE(−683/−676)

Since MUG assays on transgenic tobacco have provided evidence that the putative ERE(−683/−676) is likely active (Figure 2C), EMSAs were used to confirm DNA-protein binding. The putative binding site was reconstituted by annealing the oligonucleotides ML304 and ML305 (Figure 4A). The annealed primers ML304/ML305 showed

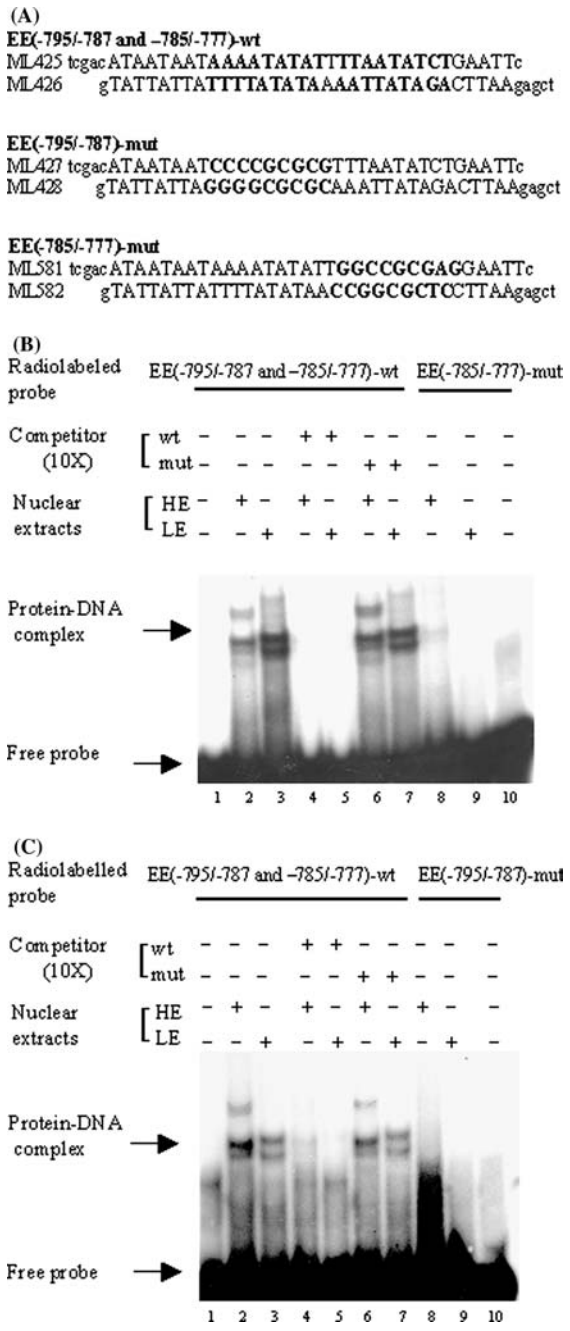

Figure 6. EMSAs on the EE(−795/−787) and EE(−785/−777) in the *SmCP* promoter region. (A) Nucleotide sequences of double-stranded oligonucleotides used in EMSAs. The mutated nucleotides in EE(−795/−787)-mut, EE(−785/−777)-mut and their corresponding sequences in EEs(−795/−787 and −785/−777)-wt are shown in bold. Upper-case letters correspond to the region of the *SmCP* promoter region with the putative EEs. Lower-case letters represent the additional sequences designed for end-labeling. (B) Interaction of nuclear proteins from *S. melongena* seedlings with EEs(−795/−787 and −785/−777)-wt or EE(−785/−777)-mut probes. Crude nuclear protein (3 μg) from circadian-regulated 5-week-old seedlings at peak (HE in lanes 2, 4, 6, 8) or low (LE in lanes 3, 5, 7, 9) *SmCP* expression was incubated with end-labeled EEs(−795/−787 and −785/−777)-wt or EE(−785/−777)-mut (lanes 8–10) in the absence (lanes 2, 3, 8 and 9) or presence of a hundredfold molar excess of unlabeled competitor, EEs(−795/−787 and −785/−777)-wt (lanes 4 and 5) or EE(−785/−777)-mut (lanes 6 and 7). Lanes 1 and 10 are free probes without addition of crude nuclear proteins. (C) Interaction of nuclear proteins from *S. melongena* seedlings with the EEs(−795/−787 and −785/−777)−wt and EE(−795/−787)-mut probes. Crude nuclear protein (3 μg) from circadian-regulated 5-week-old seedlings at peak (HE in lanes 2, 4, 6, 8) or low (LE in lanes 3, 5, 7, 9) *SmCP* expression was incubated with end-labeled EEs(−795/−787 and −785/−777)-wt (lanes 1–7) or EE(−795/−787)-mut (lanes 8–10) in the absence (lanes 2, 3, 8 and 9) or presence of a hundredfold molar excess of unlabeled competitor, EE (−795/−787 and −785/−777)-wt (lanes 4 and 5) or EE(−795/−777)-mut (lanes 6 and 7). Lanes 1 and 10 are free probes without addition of crude nuclear proteins.

binding to nuclear proteins derived from senescent (Figure 4B, lane 2) but not young fruits (Figure 4B, lane 3). In contrast, the corresponding mutant probe (Figure 4A) showed no binding to nuclear proteins from both senescent (Figure 4B, lane 8) and young fruit (Figure 4B, lane 9). Addition of a hundredfold excess of unlabeled ERE(−683/−676)-wt competed out the binding of labeled ERE(−683/−676)-wt with nuclear proteins from senescent fruits (Figure 4B, lane 4). The sequence-specificity was confirmed when unlabeled ERE(−683/−676)-mut did not compete out labeled ERE(−683/−676)-wt in binding (Figure 4B, lane 6).

In another set of EMSA experiments, the wild-type probe also showed binding to nuclear proteins from 5-week-old seedlings, irrespective of ethephon treatment (Figure 4C, lanes 2–3). In contrast, the corresponding mutant probe (Figure 4A) showed no binding to nuclear proteins from both ethephon-treated (Figure 4C, lane 8) and untreated seedlings (Figure 4C, lane 9). Addition of a hundredfold excess of unlabeled ERE(−683/−676)-wt competed out the binding of labeled ERE(−683/−676)-wt to nuclear proteins irrespective of ethephon treatment (Figure 4C, lanes 4–5). The sequence-specific binding of nuclear proteins was further confirmed when unlabeled ERE(−683/−676)-mut, consisting of the mutant ERE, could not compete against labeled ERE(−683/−676)-wt in binding (Figure 4C, lanes 6–7).

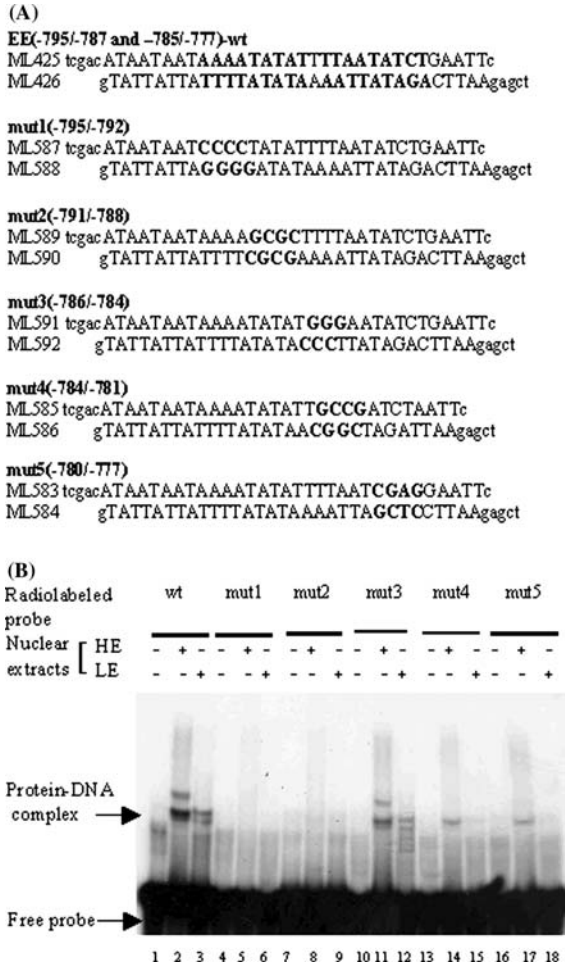

Figure 7. EMSA on the mutants of EEs in the *SmCP* promoter. (A) Nucleotide sequences of double-stranded oligonucleotides used in EMSA. The EEs(−795/−787 and −785/−777)-wt and mutant derivatives (mut1 to mut5) are shown in bold. Upper-case letters correspond to the region of the *SmCP* promoter region with the putative EEs. Lower-case letters represent the additional sequences designed for end-labeling. (B) Interaction of nuclear proteins from *S. melongena* seedlings with the EEs(−795/−787 and −785/−777)-wt and mutant derivatives (mut1 to mut5) probes. Crude nuclear protein (3 μg) from circadian-regulated 5-week-old seedlings at peak (HE in lanes 2, 5, 8, 11, 14, 17) or low (LE in lanes 3, 6, 9, 12, 15, 18) *SmCP* expression was incubated with end-labeled EEs(−795/−787 and −785/−777)-wt (lanes 1–3), mut1 (lanes 4–6), mut2 (lanes 7–9), mut3 (lanes 10–12), mut4 (lanes 13–15) or mut5 (lanes 16–18) probes. Lanes 1, 4, 7, 10, 13 and 16 are free probes without addition of crude nuclear proteins.

Analysis and identification of EEs in the SmCP *5′-flanking region*

Five deletions of the *SmCP* promoter (Figure 5A) ranging from the largest fragment (−1343/+54) to the smallest fragment (−415/+54) were analyzed to establish the molecular basis for circadian regulation with peak expression in late light. Seedlings of three independent tobacco lines transgenic for each *SmCP* promoter-*luc* construct were tested. Transgenic lines derived from pSm127 (−1343/+54), pSm128 (−1060/+54) and pSm129 (−827/+54) containing both putative EEs at −795/−787 and −785/−777 showed rhythmic luciferase expression with peak expression in late light (Figure 5C). Transgenic lines derived from plasmids pSm130 (−498/+54) and pSm131 (−415/+54), which lack the putative EEs, were arrhythmic (Figure 5C), suggesting that the two putative EEs at −795/−787 and −785/−777 confer circadian regulation with peak expression in late light.

To determine which of the two putative EEs is necessary for this expression pattern, the putative EEs were individually or doubly mutated to generate reporter constructs pSm140 to pSm142 (Figure 5B). Analysis of the transgenic plants confirmed that when either one or both EEs were mutated, circadian regulation of *luc* was completely lost, indicating the significance of both in circadian regulation (Figure 5C).

Nuclear proteins interact with EEs (−795/−787 and −785/−777)

EMSAs on the EEs were investigated using ^{32}P-dCTP-labeled probes consisting of annealed 40-mers ML425/ML426, corresponding to the two putative EEs (−795/−787 and −785/−777), and mutant oligomers ML581/ML582 and ML427/ML428, corresponding to EE(−785/−777) and EE(−795/−785), respectively (Figure 6A). Nuclear proteins were prepared from 5-week-old *S. melongena* seedlings grown in 12 h dark and 12 h light at 24 °C, harvested at peak (17:00) and at low (06:00) circadian expression. The EEs(−795/−787 and −785/−777)-wt bind nuclear proteins from seedlings harvested at peak (Figure 6B and C, lanes 2) and low expression (Figure 6B and C, lanes 3). Addition of a tenfold excess of unlabeled EEs(−795/−787 and −785/−777)-wt in the reaction competed out the binding of the corresponding labeled probe (Figure 6B and C, lanes 4–5).

Specificity of binding at EE(−785/−777) was confirmed when unlabeled EE(−785/−777)-mut competitor failed to eliminate binding of labeled EEs(−795/−787 and −785/−777)-wt (Figure 6B,

lanes 6–7). As expected, the labeled EE(−785/−777)-mut showed no binding to nuclear proteins prepared from seedlings at both peak (Figure 6B, lane 8) and low circadian expression (Figure 6B, lane 9). The sequence-specific binding of nuclear proteins to EE(−795/−787) was confirmed when labeled mutant probe EE(−795/−787)-mut did not bind nuclear proteins from seedlings at both peak (Figure 6C, lane 8) and low circadian expression (Figure 6C, lane 9). It was further confirmed when unlabeled EE(−795/−787)-mut, consisting of the mutant EE, could not compete out labeled EEs(−795/−787 and −785/−777)-wt in binding (Figure 6C, lanes 6–7).

The EEs (−795/−787 and −785/−777) show linked binding activity

Correlation of the activities of the two EEs, which are separated by three nucleotides, was investigated in EMSA using ^{32}P-dCTP-labeled probes. The wild-type probe, consisting of annealed ML425/ML426 (−795/−787 and −785/−777) (Figure 7A), binds nuclear extracts at peak (Figure 7B, lane 2) as well as low circadian expression (Figure 7B, lane 3). The labeled mutant probes mut1 (ML587/ML588) and mut2 (ML589/590) (Figure 7A) which are partial mutations of EE(−795/−787) failed to bind either nuclear proteins (Figure 7B, lanes 5–6 and 8–9). Similarly, mut4 (ML585/ML586) and mut5 (ML583/584) (Figure 7A) which are partial mutations of EE(−785/−777) failed to bind some nuclear proteins at peak expression (Figure 7B, lanes 14–17) and there was no binding to nuclear proteins at low expression (Figure 7B, lanes 15–18). In contrast, mut3 (ML591/ML592) containing the mutated nucleotides (TTT) between the two EEs did not affect binding. Like the wild-type probe, it binds nuclear protein extract at peak (Figure 7B, lane 11) as well as low circadian expression (Figure 7B, lane 12).

DNase I footprinting analysis of EEs (−785/−777 and −795/−787)

Subsequently, DNase I footprinting was carried out on the coding stand of the *SmCP* promoter to confirm the binding of the putative EEs to nuclear proteins. Incubation of the ^{32}P-end-labeled −827/−706 *SmCP* promoter strand with nuclear proteins from leaves at peak or at low circadian expression revealed strong protected areas on the top strand from −795 to −781 and −771 to −762, and weaker protection in the adjacent regions between −807 to −794 and −782 to −770 (Figure 8).

Discussion

Although cysteine proteinase genes have been cloned from many plant species and shown to exhibit diverse expression patterns, reports on the characterization of their corresponding promoters in transcriptional regulation are limited. The promoter of *EPB-I*, a barley cysteine proteinase gene which is induced by gibberellins and repressed by abscisic acid, contains a gibberellin response element (GARE) as deduced by deletion and mutational analysis (Cercos *et al*., 1999). Unlike tobacco in which two genes, *CYP-7* and *CYP-8*, encode cysteine proteinases, it is encoded by a single gene in brinjal (Xu and Chye, 1999), making its expression analysis simpler. Given the significance of *SmCP* in protein degradation likely in PCD-related events and that it is ethylene-inducible and circadian-regulated, an understanding on its control of expression was sought. Hence steps were taken in this study to identify and characterize its *cis*-elements. We analyzed ethephon-treated and untreated transgenic tobacco seedlings expressing various *SmCP* promoter-*GUS* constructs to define the regions conferring ethylene-responsive *SmCP* expression. We confirmed that the plants expressing constructs pSm13, pSm14 and pSm16 containing the putative ERE(−141/−134) alone show lack of ethephon induction despite its close identity to the ERE of *GST1* (ATTTCAAA). This is consistent with our previous observations (Xu *et al*., 2003) in the lack of this ERE in binding nuclear proteins in EMSA and in DNase I footprinting. Possibly ERE(−141/−134) is non-functional or these deletion constructs lack further upstream elements that act synergistically with ERE(−141/−134). Deletion analysis also suggest that ERE(−355/−348) mediates a low-level ethylene-responsiveness which increases in the presence of an additional ERE(−683/−676). ERE(−355/−348) and its 5′-flanking nucleotide (in italics) *A*AATTCAAG share greater homology (nucleotide mismatch underlined) to that of tomato *E4* (*A*AATTCAAA)

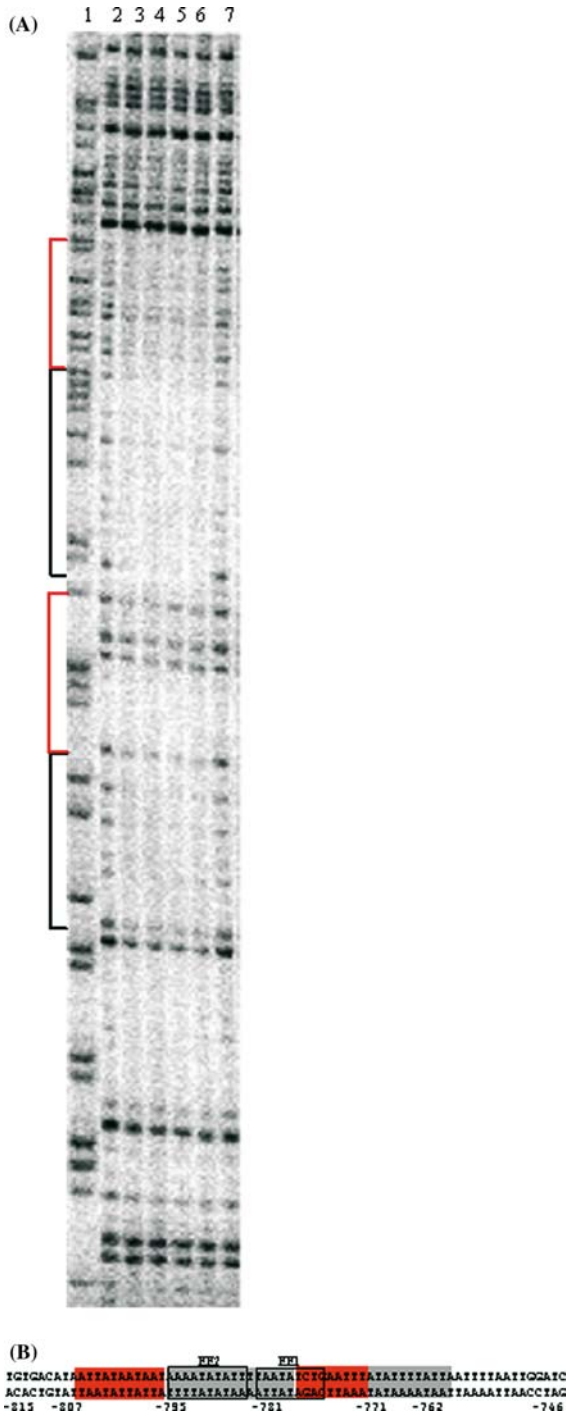

Figure 8. *In vitro* DNase I footprinting analysis of the *SmCP* promoter using ^{32}P-dCTP-labeled probes containing the −827/−706 region. (A) DNase I protection using nuclear extract from leaves at peak and low circadian expression. Lanes: 1, G/A, Maxam-Gilbert sequencing (G + A) reactions of the labeled promoter fragment used in localization of the protected region; 2 and 7, DNase I digestion pattern with BSA; 3 and 4, crude nuclear protein (100 and 150 μg respectively) from leaves with peak *SmCP* expression; 5 and 6, crude nuclear protein (100 and 150 μg respectively) from leaves with low *SmCP* expression. Regions protected from DNase I digestion are denoted in black (strong protection) and red (weak protection). (B) DNA sequence of protected regions detected in (a). The *SmCP* promoter sequence from −815 to −746 is shown. Regions of strong protection are highlighted in grey and those of weak protection are in red.

than to *GST1* (GATTTCAAA). Similarly, ERE(−683/−676) *A*A<u>T</u>TTCAAA also shares a significant homology to that of tomato *E4*.

Nuclear proteins have been shown to interact specifically with the EREs at the promoters of tomato *E4* (Montgomery *et al*., 1993; Coupe and Deikman, 1997) and carnation *GST1* (Itzaki *et al*., 1994; Maxson and Woodson, 1996). Senescent fruits have high endogenous ethylene in comparison to young fruits. Unlike the ERE of *E4* which binds only nuclear proteins of unripe but not ripe fruits (Montgomery *et al*., 1993), the double-stranded oligos corresponding to ERE(−355/−348) and ERE(−683/−676) of *SmCP*, interact specifically with the nuclear proteins from senescent, but not young, fruits in EMSAs. Also, the EREs at −355/−348 and −683/−676 bind nuclear proteins from both ethephon-treated and untreated seedlings. The specificities of the interactions were verified by competition experiments in which only those oligomers, consisting of the conserved AATTTCAA<u>A</u>/G sequence, were able to compete in binding. A similar pattern of binding has been previously reported with the ERE of carnation *GST1*, which binds nuclear proteins from both senescent as well as presenescent petals (Maxson and Woodson, 1996). Possibly, different transcriptional factors bind to the same ERE under varying physiological conditions. The differences in the binding pattern of fruit and seedling nuclear proteins to the *SmCP* EREs indicate that organ-specific factors may participate in binding.

Computational studies have identified a 9-nucleotide conserved EE motif (AAAATATCT) in the promoters of the circadian-regulated *Arabidopsis* genes that show peak expression in late light (Harmer *et al*., 2000). The promoter of tobacco *ZGT* that is under such circadian

regulation contains two EEs (AAAATATCT at −178/−186 and AATATCT at −231/−237) ca. 50 nucleotides apart in an antisense orientation (Xu and Johnson, 2001). In contrast, those at the *SmCP* promoter are separated by only three nucleotides and are unidirectional. Deletion analysis and mutation experiments carried out in this study revealed that the two EEs of *SmCP* are functionally active and are important in mediating circadian regulation of *luc*. EMSAs using wild-type and various mutant probes within the EEs (−795/−777) suggest that they show co-operative activities. EMSAs with nuclear proteins harvested at peak or low-circadian expression suggest that both EEs show multiple DNA-protein complexes, implicating that two or more proteins either bind independently or form a protein complex *via* protein–protein interactions before binding to the same EE. Regions of strong and weak binding observed overlapping the location of the EEs on DNase I footprinting, is likely due to the conformation of the multi-protein complexes interacting on the EEs. Such complexes in EMSAs have been previously reported in the circadian-regulated *CAB2* (*lhcb1*1*) promoter that shows peak expression in early light (Carre and Kay, 1995). Occurrence of protein–protein interactions are not uncommon in circadian control (Yanovsky and Kay, 2001) and such interacting proteins including ZEITLUPE (ZTL) (Somers *et al*., 2000) and FLAVIN-BINDING, KELCH REPEAT, F BOX 1 (FKF1) (Nelson *et al*., 2000) have already been identified in *Arabidopsis*. The sequence to which the transcription factors bind as identified by DNase I footprinting may extend beyond the actual EE consensus sequence if a large multi-transcription factor complex is interacting. It is known that the transcription factor TIIFD binds to the consensus TATAAAA sequence (TATA box), however, it protects a larger region of about 35 bp sequence around the TATA box in DNase I footprinting (Lewin, 1997). The ERE motif (AAATTCAAA) of the tomato *E4* promoter also lies within a larger 20 bp region, protected from DNase I digestion (Montogomery *et al*., 1993).

The binding pattern of nuclear proteins to *SmCP* EEs at peak circadian expression varies from that at low, suggesting the possibility of different transcription factors binding to the same motifs under varying conditions. According to the model proposed by Alabadi *et al*. (2001) the Myb-related transcription factors CCA1 and LHY bind to an EE (AAAATATCT) in the *TOC1* promoter and repress its expression. When the levels of CCA1 and LHY decline during the day, TOC1 accumulates and activates the transcription of *CCA1* and *LHY*, forming the base for transcriptional feedback loop. Several other genes and proteins involved in circadian clock regulation have been identified and investigations on their interaction should provide a more detailed molecular understanding of clock regulation.

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