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Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene

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We began characterization of the protein-DNA interactions necessary for UV light induced transcriptional activation of the gene encoding chalcone synthase (CHS), a key plant defense enzyme. Three light dependent *in vivo* footprints appear on a 90 bp stretch of the CHS promoter with a time course correlated with the onset of CHS transcription. We define a minimal light responsive promoter by functional analysis of truncated CHS promoter fusions with a reporter gene in transient expression experiments in parsley protoplasts. Two of the three footprinted sequence 'boxes' reside within the minimal promoter. Replacement of 10 bp within either of these 'boxes' leads to complete loss of light responsiveness. We conclude that these sequences define the necessary *cis* elements of the minimal CHS promoter's light responsive element. One of the functionally defined 'boxes' is homologous to an element implicated in regulation of genes involved in photosynthesis. These data represent the first example in a plant defense gene of an induced change in protein-DNA contacts necessary for transcriptional activation. Also, our data argue strongly that divergent light induced biosynthetic pathways share common regulatory units.

Key words: chalcone synthase/inducible transient expression/*in vivo* footprinting/light regulation/parsley protoplasts

Introduction

Active defense mechanisms in plants are mediated largely by the induced accumulation of protective compounds in response to environmental stress. Various classes of flavonoids, for example, function as UV absorbants, pigments and, in legumes, phytoalexins (McClure, 1975; Hahlbrock and Grisebach, 1979; Ebel, 1986). The gene encoding chalcone synthase (CHS), the first committed enzyme of flavonoid biosynthesis, is rapidly activated at the transcriptional level in response to stress caused by UV irradiation (Chappel and Hahlbrock, 1984; Dangl *et al.*, 1989, for review).

In darkgrown parsley (*Petroselinum crispum*) cell suspension cultures, irradiation with UV light or UV containing white light leads to a massive increase in CHS transcriptional activity 2-4 h after the onset of treatment (Chappell and Hahlbrock, 1984; Ohl *et al.*, 1988). The series of events in suspension cultured cells leading to vacuolar deposition of UV absorbing flavonoids parallels that occurring in epidermal cells of developing or light-grown parsley leaves (Jahnen and Hahlbrock, 1988; Schmelzer *et al.*, 1988). Blue light and phytochrome modulate CHS gene activation (Duell-Pfaff and Wellman, 1982; Bruns *et al.*, 1986; Ohl *et al.*, 1988). Thus, three photoreceptor systems may be involved in signal perception and transduction. There is, however, a paucity of detailed knowledge regarding the regulatory processes governing these events. Earlier analysis of the heterologous *Antirrhinum majus* CHS gene in transgenic tobacco suggested that 1.1 kb of promoter was sufficient to render a reporter gene light responsive (Kaulen *et al.*, 1986).

Cloning and structural analysis of two allelic parsley CHS genes (Herrmann *et al.*, 1988) and development of a light responsive homologous protoplast system for gene transfer (Dangl *et al.*, 1987) allowed us to begin characterization of light induced protein-DNA interactions on the parsley CHS promoter.

Results

Light induced in vivo DNA footprints on the CHS promoter

We analyzed light induced protein-DNA interactions on the CHS gene from approximately +40 to -245 with respect to the transcription start site. Figure 1 shows results of *in vivo* DNA footprinting experiments (Church and Gilbert, 1984) of the parsley CHS-1 allele promoter from around -100 to -245. Schematic interpretation of these data is shown in Figure 2. Clear local changes in DNA methylation protection are apparent on both the coding and non-coding strands throughout a 6.5 h time course beginning at 2 h after the onset of irradiation. The appearance of these footprints is strongly correlated to the onset of maximal CHS transcription (Chappell and Hahlbrock, 1984; Ohl *et al.*, 1988).

Three regions of sequence are defined by these results. Region I, at around -140, is fairly large and may be centred on the twice reiterated sequence 5'-AACCT-3'. The presence of a strongly hyper-reactive A residue on the coding strand (-138) is worthy of note. This may imply local helix distortion of the minor groove in region I, since adenine residues at N-7 positions in the minor groove are normally nearly inaccessible to methylation (Siebenlist and Gilbert, 1980; DiCapua and Müller, 1987). Region II contains an octamer with perfect dyad symmetry (5'-CCACGTGG-3') at position -165; while region III is a degenerate repeat of region II at position -230 (Figure 2b). Putative protein contacts in sequences shared by regions II and III are identical, characterized on both strands by hypomethylation of the two internal G residues and hypermethylation of the external G residue (Figure 2b). From these data we conclude that light induced changes in protein-DNA interactions between -100 and -245 are distinct, coordinate in their appearance and correlated with the onset of CHS transcription.

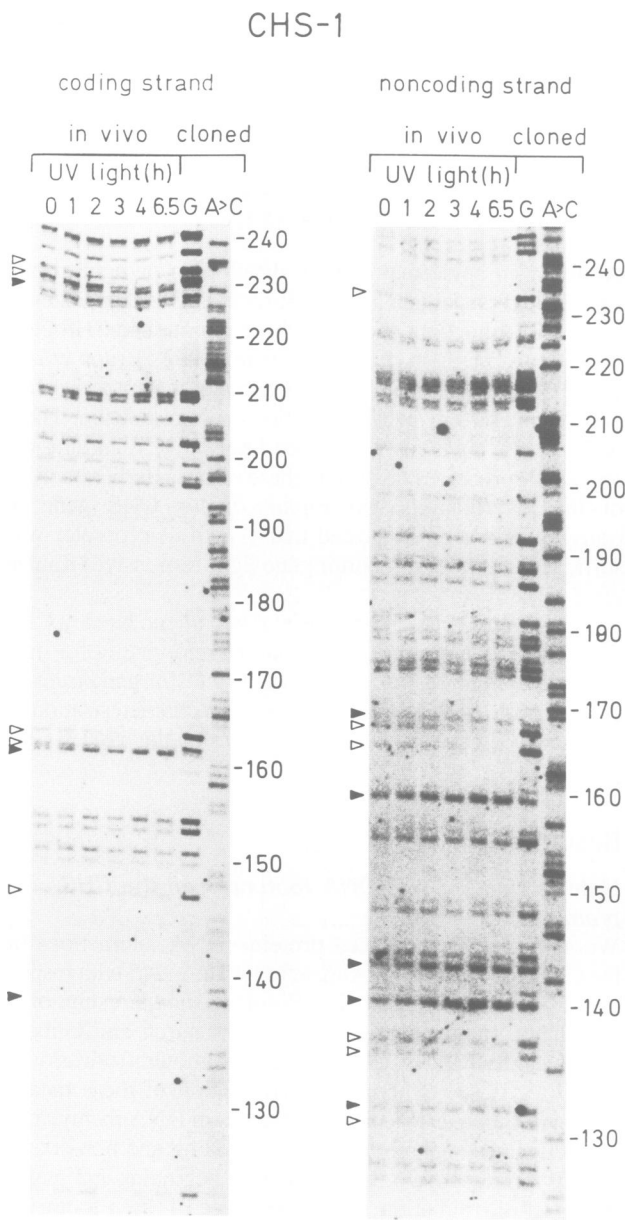

Fig. 1. Kinetics of UV light induced changes in the reactivity to DMS *in vivo* of CHS 5′ promoter sequences. Genomic DNA sequence G ladders of a region of the parsley CHS-1 allele spanning −130 to −245 upstream of the transcription start site are shown. Light induced changes in the reactivity to DMS *in vivo* are analysed at indicated times after the onset of light induction. Protections from and enhancements of DMS methylation are shown as open and closed triangles, respectively. Numbers on the right refer to the position of bases relative to the transcription start site. Reference sequence ladders for the G and A + C reactions of *in vitro* treated DNA are also shown. The single-stranded DNA probes used to visualize the genomic sequence are shown as arrows in Figure 2a.

Functional role of footprints addressed through transient gene expression

We assessed the functional significance of the three DNA footprints through light induced transient gene expression in transformed parsley protoplasts. A CHS-1 promoter fragment was fused translationally to the *Escherichia coli* glucuronidase (GUS) reporter gene (Jefferson *et al.*, 1986, 1987) (Figure 3a). Various 5′ endpoint derivatives of this were constructed and transferred to freshly prepared parsley protoplasts (Dangl *et al.*, 1987) which were then immediately irradiated for 9 h. This time point reflects the known maximum for CHS expression in parsley protoplasts (Dangl *et al.*, 1987) as well as the maximum for light induced GUS activity of CHS promoter constructs (our unpublished data).

Promoter maps and data from two typical GUS assays are given in Figure 3b. These data show that a CHS promoter fragment spanning from −100 to −226, containing footprint regions I and II, is light responsive in conjunction with the cognate promoter up to −100. However, sequences further 5′, which include region III, clearly increase both induced and uninduced levels of GUS expression. Importantly, deletion of all footprinted regions, leaving 100 bp of promoter, results in uninducible basal levels of GUS activity. Mutations in 10 bp boxes (Figure 2b) containing the footprints of either region I or region II, in the context of the minimal light responsive construct 061, abolish inducibility. These functional data prove that both boxes I and II are necessary for a light response in the context of the minimal parsley CHS promoter (061).

Discussion

Box II homology occurs in various inducible gene systems

Are the induced protein−DNA interactions at regions I and II of specific relevance to light/UV light responsiveness? We could find no significant homologies in the EMBL databank to a 13 bp sequence containing box I. Box II, however, seems to be conserved, to some extent, in CHS genes from other species which are known to be light regulated (Table I). It can also be found in two other UV activated parsley genes, one of unknown function and one which encodes phenylalanine ammonia-lyase, a key enzyme in the general phenylpropanoid pathway (R.Lois, W.Schulz and K. Hahlbrock, in preparation). The box II sequence is not found in another light regulated gene of the general phenylpropanoid pathway, 4-coumarate: CoA ligase (Douglas *et al.*, 1987). Moreover, a stringent computer search (9/10 minimum homology) revealed the surprising find that a 10 bp sequence containing the octamer of box II is significantly conserved in promoters from genes not known to be light regulated. Its occurrence in genes under anaerobic (*adh*), developmental (*patatin*) or pathogenic (*rolbc*) control argues strongly against a strictly UV or light dependent role for factors binding to it.

Overlapping control networks among light regulated gene systems

The conservation of the CHS box II sequence, in both composition and position, among a plethora of ribulose bisphosphate carboxylase small subunit (SSU) genes is also striking (Table I). Its functional role in SSU regulation has not been directly addressed. Recently, however, a nuclear factor binding an oligonucleotide containing this sequence was identified from light-grown and dark-adapted leaves of tomato and *Arabidopsis* (Giuliano *et al.*, 1988). These authors lack functional data regarding the role of this sequence element (termed the 'G box') in SSU regulation, although a G box homology as part of a 132 bp element isolated from the tobacco *Cab-E* gene does confer photoregulated expression onto a heterologous promoter in transgenic plants (Castresana *et al.*, 1988). SSU promoter

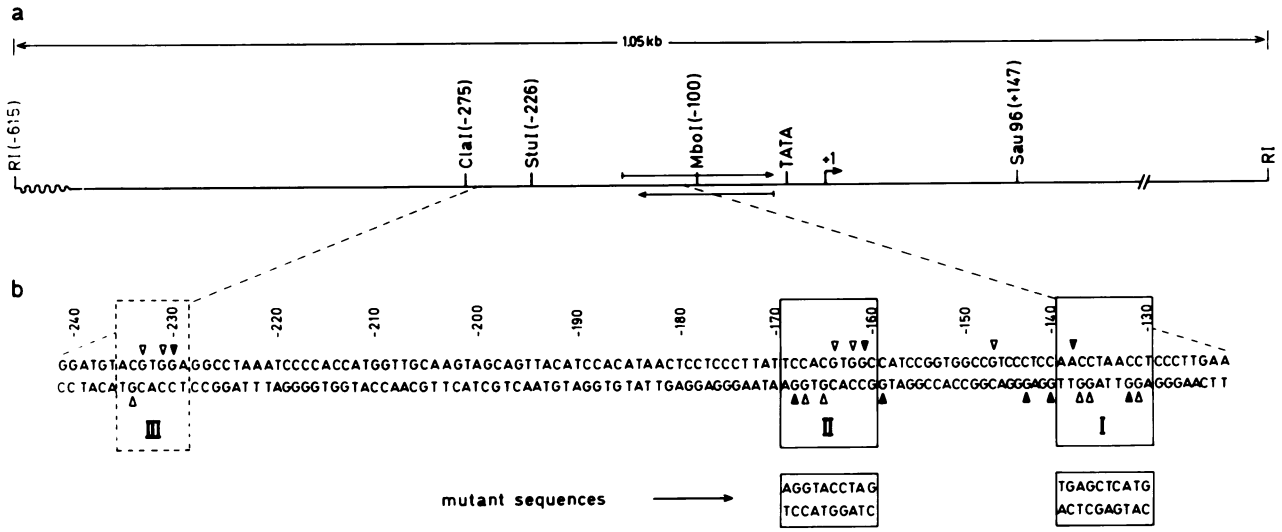

Fig. 2. Footprinted region of the parsley CHS gene. (**a**) Schematic diagram of the CHS-1 allele promoter. A 1.05 kb *Eco*RI fragment containing 615 bp upstream from the transcription start site is shown. The wavy line indicates sequences derived from a potential transposition event characteristic of the CHS-1 allele (Herrmann *et al.*, 1988). Landmark restriction sites and the positions of probes used (arrows) for genomic sequencing are given. (**b**) CHS-1 promoter sequence in the footprint regions. Data from Figure 1 are interpreted as follows: open triangles, hypomethylated G residues; closed triangles, hypermethylated G or A residues. The coding strand is on top. Roman numerals indicate sequence boxes defined in the text. The boundaries of box III were deduced by comparison with box II. Mutant sequences are those used to replace nucleotides within boxes I and II. The site at −100 is *Mbo*II not *Mbo*I.

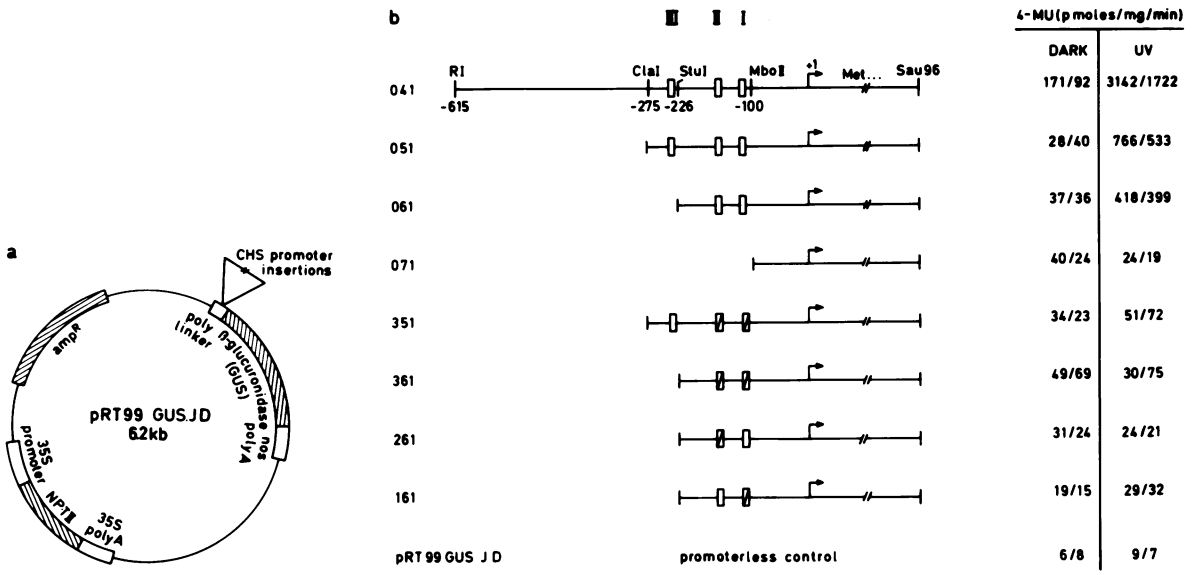

Fig. 3. Light induced transient expression from truncated and site mutated CHS promoter derivatives (**a**) The base vector, pRT99.GUS.JD, contains the *E.coli uidA* gene (Jefferson *et al.*, 1986, 1987) cloned into the polylinker of pRT99 (Töpfer *et al.*, 1988). (**b**) CHS promoter mutants and the results from two GUS assays. Construct designations are on the left, and positions of restriction sites used in their construction are shown on the top line. Open boxes represent wild-type sequences within the inducible footprint regions; boxes with a diagonal line through them represent mutants with sequence replacements shown in Figure 2b. Expression data are given as specific glucuronidase activity measured as the rate of conversion of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone (4-MU). Data from two experiments are shown, each experiment representing the averaged results of two separate transformations.

architecture has been extensively analysed both in transgenic plants and via *in vitro* analysis of DNA binding activities (see Kuhlemeier *et al.*, 1987a; Manzara and Gruissem, 1988; for reviews). The SSU homology with the CHS box II (G box) is located between sequences known to form part of a functionally redundant complex of *cis* elements, namely directly downstream from box II* (Green *et al.*, 1987; Kuhlemeier *et al.*, 1987b). In pea SSU promoters, the larger context of the G box is a 360 bp fragment conferring developmental and cell specific expression, as well as light responsiveness (see Kuhlemeier *et al.*, 1987a for review; Aoyagi *et al.*, 1988).

Why has no functional significance in SSU regulation been ascribed to this sequence? We note the obvious difference between transgenic plants, used in most SSU analyses, and suspension cultured cells. Also, assays of steady state mRNA levels in transgenic plants (light-growth, dark-adaptation and re-exposure to light) have revealed complex, overlapping

Table I. A comparison of box II sequences from various plant genes.

Plant gene	Sequence; cf. parsley CHS box II	Position	Reference
CHS gene from parsley			
Petroselinum crispum			
box II	TCCACGTGGC	−169	Herrmann *et al.* (1988)
box III rev.	. . . . . a c a	−240	Herrmann *et al.* (1988)
CHS genes from other plants			
Zea mays	c	−90	Niesbach-Klösgen (1987)
Antirrhinum majus	a . . . . . a t .	−70	Sommer and Saedler (1986)
Glycine max			
cs1	g . . . . . a . t .	−100	R.Wingender-Drissen (unpublished data)
cs1	. . t . . . . a . .	−140	R.Wingender-Drissen (unpublished data)
cs2	. . . . . a t	−395	R.Wingender-Drissen (unpublished data)
Petunia hybrida (V30)			
gene A	g t . . . . . c .	−91	J.Mol and R.Koes (unpublished data)
gene J	. a . . . . . a t	−70	J.Mol and R.Koes (unpublished data)
Petunia hybrida (Red Star)	. a . . . . . a t	−110	J.Reif (unpublished data)
Other plant genes			
Petunia hybrida			
rbcS 301	. . . . .	−226	Tumer *et al.* (1986)
Pisum sativum			
rbcS 3C	. . . . .	−182	Fluhr *et al.* (1986)
Arabidopsis thaliana			
rbcS 1A	. . . . .	−235	Timko *et al.* (1985)
Arabidopsis thaliana			
Adh rev.	. . . . .	−200	Chang and Meyerowitz (1986)
Glycine max			
rbcS 1	. . . . . t .	−208	Grandbastien *et al.* (1986)
rbcS 4	. . . . . t .	−208	Grandbastien *et al.* (1986)
Nicotiana plumbaginifolia			
rbcS 8B	. a	−231	Poulsen *et al.* (1986)
Nicotiana tabacum			
rbcS	. a	−220	Mazur and Chiu (1985)
Petunia hybrida			
rbcS 611	g	−190	Tumer *et al.* (1986)
Pisum sativum			
rbcS 3.6	. a	−212	Herrera-Estrella *et al.* (1984)
rbcS 8.0	. a	−210	Timko *et al.* (1985)
rbcS E9	. a	−218	Coruzzi *et al.* (1984)
rbcS 3A	. . . . . a	−212	Fluhr *et al.* (1986)
Solanum tuberosum			
rbcS 1	. a	−200	C.Fritz (unpublished data)
Solanum tuberosum			
patatin	. . . . . c . .	−100	Rosahl *et al.* (1986)
Nicotiana glauca			
rolbc	. . . . . c . .	−100	Forner *et al.* (1986)

groups of *cis* elements needed for high levels of, or tissue specific, expression. In our experiments we assessed light induced changes in protein−DNA interaction through *in vivo* footprinting in dark-grown cells, thus monitoring inductive events at the template. Importantly, our interpretation of the functional necessity of box II (when present with box I) was made in the context of the comparatively simple CHS minimal promoter. It could be argued that compensating functional overlap has masked the significance of the G box in previous SSU analyses (Kuhlemeier *et al.*, 1987a). We also speculate that the potential control mechanisms shared by CHS and SSU could be mediated in part by the blue light response known to be operative in both systems (Fluhr *et al.*, 1986; Ohl *et al.*, 1988).

For CHS, as with SSU, we expect ultimately to uncover layers of functional redundancy, since addition of sequences from −275 to −615 onto construction 351 restores a level of inducibility similar to the minimal promoter construct 061 (not shown). Whether this is due to the influence of one or more upstream elements acting on region III or the presence of a second system controlling light responsiveness is under investigation. We note in this regard that the level of UV light induced GUS expression observed with construct 351 (2- to 3-fold) is very consistent.

Functional complexity in CHS promoters is foreshadowed by recent analysis of the *A.majus* CHS promoter in parsley protoplasts (Lipphardt *et al.*, 1988). These authors demonstrated the presence of three functionally distinct sequence regions: the first, a TATA proximal fragment of 160 bp is a minimal light responsive element which contains a sequence with homology to box II (see Table I); the second, directly adjacent, is a light dependent enhancer; the third

is an upstream general enhancer located between −564 and −661.

We propose that box II (G box) related sequences bind factors which may be differentially utilized during a multiplicity of stress responses, as well as during light regulation. Combinational diversity of *cis* elements could result both from shuffling of template sequences and through divergence in the non-DNA contacting portions of proteins binding to them, generating complex arrays of regulatory networks. Also, protein−protein interactions, mediated through *cis* element shuffling, may generate the refined transcriptional regulation operating during various plant stress reactions.

Materials and methods

Genomic footprinting

Cultured parsley cells, grown for 6 days in the dark, were continuously illuminated for 1, 2, 3 4 and 6.5 h with light from Phillips TL20W18 lamps emitting wavelengths between 300 and 400 nm. A 200 ml aliquot of cell suspension culture from each time point was treated with 0.5% (v/v) dimethylsulphate (DMS) final concentration for 1 min at 26°C. Reactions were terminated by 10-fold dilution of the medium with ice-cold water and the cells retrieved in a Büchner funnel on an S and S membrane filter. Cells were washed once with 200 ml ice-cold water and frozen in liquid nitrogen. Genomic DNA was prepared from the cells and recovered from CsCl gradients. Genomic DNA (600 μg) from each time point was cut with *Eco*RI. The digested DNA was size separated on a linear sucrose/salt gradient (10−30% sucrose/0.1 M NaCl in 10 mM Tris−HCl, pH 7.5, 20 mM EDTA). Fractions containing the 1.05 kb *Eco*RI CHS-1 promoter fragment were pooled, dialysed against 10 mM Tris, pH 7.5, 1 mM EDTA and ethanol precipitated. The enrichment for the CHS-1 promoter fragment after this procedure was estimated to be ~50-fold. The enriched DNA was then cut with *Eco*RV (at position −45) to create the reference point for the displayed genomic G ladder (Figure 1), extracted with phenol/$ChCl_3$, treated with piperidine and recovered by lyophilization. Approximately 5 μg DNA from each time point were loaded onto 0.5 mm thick and 80 cm long sequencing gels. Electroblotting, UV cross-linking of the DNA to Genescreen (NEN), hybridization and washing of the filters was essentially as described by Church and Gilbert (1984). Probe synthesis (Church and Gilbert, 1984) had the following modifications: two synthetic 17mer oligonucleotides homologous to either the coding (5′ end at −162) or non-coding (5′ end at −45) strand were annealed to an M13 single-stranded template containing the 1.05 kb *Eco*RI fragment from CHS-1 and elongated in reactions with 250 μCi of [^{32}P]dATP (Amersham), 3000 Ci/mmol. Synthesis products were restriction digested with either *Eco*RV (coding strand probe) or *Hae*III (non-coding strand probe), size separated on a 6% polyacrylamide gel containing 8 M urea, and the single-stranded probes recovered by isotachophoresis (Öfverstedt *et al.*, 1984).

Plasmid constructions

Construct 041 was generated by cloning the Klenow filled *Eco*RI−*Sau*96 fragment from CHS-1 into the Klenow filled *Xba*I site of the base vector pRT99 (Töpfer *et al.*, 1987), leading to an in-frame translational fusion including 20 amino acids from the CHS protein and 10 amino acids encoded by the polylinker of the base vector. All deletion derivatives were constructed by standard techniques. The target for site-directed mutagenesis was the *Cla*I−*Sau*96 CHS-1 fragment cloned into the pMa/c vector system (a gift from Dr P.Stanssens, Plant Genetic Systems, Ghent, Belgium). Mutants were generated (Kramer *et al.*, 1984) using a 40mer oligonucleotide containing 15 bases of homology on either side of the 10-bp mismatch. Single or double mutants were shuttled back into expression plasmids as *Stu*I−*Xba*I fragments. All mutations and translation fusions were verified by dideoxy chain termination sequencing (Sanger *et al.*, 1977).

Light induced transient expression assays

Supercoiled plasmid DNA (20 μg/10^6 protoplasts) was transferred into freshly prepared parsley protoplasts (Dangl *et al.*, 1987) using the polyethylene glycol (PEG) method (Krens *et al.*, 1982; Hain *et al.*, 1985). Each transformation assay of 2×10^6 protoplasts was split and placed into two 3 ml plates, one kept in the dark, the other immediately irradiated. After 9 h, protoplasts were harvested, frozen in liquid nitrogen, crude protein extracts prepared and GUS activity assayed (Jefferson *et al.*, 1987). Bradford assays (Bio-Rad) were used for protein determination.

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Note added in proof

Sequence analysis of all four members of the SSU gene family from *Arabidopsis* has recently been published [Krebbers,E., Seurinck,J., Herdies,L., Cashmore,A.R. and Timko,M.P. (1988) *Plant Mol. Biol.*, **11**, 745–760]. Perusal of these sequences shows that two more *Arabidopsis* SSU genes, other than the one included in Table I, have either perfect or very high homology with our box II sequence. Interestingly, the fourth SSU gene, which lacks a block of 43 bp relative to the others containing this sequence is nevertheless expressed in leaf tissue.