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Exonic sequences are required for elicitor and light activation of a plant defense gene, but promoter sequences are sufficient for tissue specific expression

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The parsley *4CL-1* gene encodes 4-coumarate:CoA ligase, a key enzyme of general phenylpropanoid metabolism. As well as being transcriptionally activated by such stresses as pathogen infection, UV-irradiation, and wounding, expression of *4CL-1* is developmentally regulated. In this paper we present evidence that *4CL-1* *cis*-acting elements which control stress-induced and developmental expression are physically separated. The ability of a series of *4CL* gene constructions to respond to elicitor and light in stably or transiently transformed parsley cells was tested. While inducible expression was observed from all templates in which the *4CL-1* structural gene was fused to the *4CL-1* promoter, fusions of the promoter to the GUS reporter gene were completely unresponsive. The element(s) required for responsiveness appear to be exonic, since *4CL-1* introns and 3′ flanking DNA had no effect on inducibility. Furthermore, this unconventional regulatory mode operates in transgenic tobacco plants, where we show that a *4CL-1* promoter fragment specifies correct cell-specific expression when fused to GUS yet is unresponsive to elicitor and light.

Key words: cis-acting elements/4-coumarate:CoA ligase/stress induction/parsley/transgenic plants

Introduction

An almost ubiquitous feature of plant responses to environmental insult is the induced accumulation of protective proteins and secondary metabolites. Activation of phenylpropanoid metabolism is a common defense response to attempted pathogen attack, light stress and wounding, among others. Through the activities of general phenylpropanoid pathway enzymes and end-product specific branch pathways, an astounding array of secondary metabolites are elaborated from phenylalanine. As well as antimicrobial phytoalexins and UV-protective flavenoids, phenylpropanoid derivatives also act as flower and fruit pigments, activators or inhibitors of symbiotic plant-microbe interactions, and insect repellents and attractants. Moreover, phenylpropanoid derivatives such as lignin and suberin are also critical constituents of many differentiated cell types. Thus, it is not surprising that the structure and regulatory strategies of phenylpropanoid genes have received great attention (Ebel, 1986; Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1989; Lamb *et al.*, 1989; Dixon and Harrison, 1990; and Dangl, 1991 are a selection of recent reviews). This research effort is due, in part, to the seemingly paradoxical requirement for both exquisite temporal and cell-type specific accumulation of specific functional end-products, and the general need of plants to respond rapidly to certain environmental stimuli in a cell-type independent manner.

Genes encoding key enzymes of general phenylpropanoid and flavonoid metabolism have been cloned from a number of species (listed by Mol *et al.*, 1988; Dixon and Harrison, 1990). Analysis of stress activated and cell-type specific expression and regulatory *cis*-acting sequences has begun (Kaulen *et al.*, 1986; Dron *et al.*, 1987; Lipphardt *et al.*, 1988; Bevan *et al.*, 1989; Liang *et al.*, 1989a,b; Lois *et al.*, 1989; Schulze-Lefert *et al.*, 1989a; 1989b; Block *et al.*, 1990; Lawton *et al.*, 1990; Ohl *et al.*, 1990; Schmid *et al.*, 1990; van der Meer *et al.*, 1990). These and subsequent analyses will serve as paradigms for regulatory strategies which must incorporate specific developmental cues with the environmental stress signals superimposed upon them.

Much interest has focused on genes encoding either the first enzymatic step of the general phenylpropanoid pathway, L-phenylalanine ammonia lyase (*PAL*) or the committed step in flavonoid biosynthesis, chalcone synthase (*CHS*). Equally intriguing are the genes encoding 4-coumarate:CoA ligase (*4CL*), since its activity is the branchpoint from the general phenylpropanoid pathway into several end-product specific pathways. To date, analysis of *4CL* gene structure and regulation is limited to parsley and potato (Douglas *et al.*, 1987; Becker-Andre *et al.*, 1991).

In the parsley system, cultured cells treated with fungal elicitor derived from the fungus *Phytophthora megasperma* f.sp. *glycinea* (*Pmg*) excrete furanocoumarin phytoalexins (Tietjen *et al.*, 1983; Scheel *et al.*, 1986; Hahlbrock and Scheel, 1989). As well, irradiation of these cells with UV-containing white light (referred to throughout as 'light') leads to synthesis and vacuolar deposition of UV-absorbing flavonoids (Hahlbrock *et al.*, 1982). Control of these biosynthetic steps is primarily transcriptional for all phenylpropanoid and flavonoid genes analyzed (Chappell and Hahlbrock, 1984; Cramer *et al.*, 1985; Douglas *et al.*, 1987; Lawton and Lamb, 1987; Lois *et al.*, 1989). A similar series of events is induced in parsley plants either by inoculation of cotyledons with fungal spores or by light treatment of etiolated leaves (Jahnen and Hahlbrock, 1988; Schmelzer *et al.*, 1988). *In situ* hybridization showed that *PAL* and *4CL* mRNAs accumulate in a cell-type independent manner around sites of attempted *Pmg* infection, and in the epidermal

cells of light-treated leaves (Schmelzer *et al.*, 1989). As well, *PAL* and *4CL* mRNAs accumulate locally around wound sites on parsley leaves. These examples of environmentally induced *4CL* expression are layered atop complex cell-type specific and developmentally regulated *4CL* activity (Jahnen and Hahlbrock, 1988; Schmelzer *et al.*, 1989; Becker-Andre *et al.*, 1991; Dangl, 1991; Hauffe *et al.*, 1991). Detectable *4CL* mRNA accumulates preferentially in vascular tissues, oil-duct cells and epidermal cells in parsley leaves (Schmelzer *et al.*, 1989).

What are the *cis*-acting elements mediating *4CL* expression in response to diverse environmental and developmental signals? We present evidence here that gene-internal sequences are required, in addition to promoter sequences, for the high level *4CL* expression induced by fungal elicitor or light treatment in both transformed parsley suspension cultured cells and transgenic tobacco plants. In contrast, however, we also show that *4CL* promoter sequences alone are sufficient to direct cell-type specific expression of a receptor gene. This separation of *cis*-acting sequences responding to developmental and environmental signals is contrasted to recent analyses of heterologous *PAL* and *CHS* genes, where promoter sequences control both modes of expression in transgenic tobacco (Bevan *et al.*, 1989; Liang *et al.*, 1989b; Ohl *et al.*, 1990; Schmid *et al.*, 1990; van der Meer *et al.*, 1990).

Results

4CL promoter derivatives in stably and transiently transformed parsley suspension cells

Parsley contains two highly homologous (>95%) *4CL* genes, *4CL-1* and *4CL-2*, which are both activated by either fungal elicitor or light treatment (Douglas *et al.*, 1987; Lozoya *et al.*, 1988). Since it is therefore unlikely that differential regulation of the two promoters is responsible for *4CL* activation by different stimuli, we concentrated on the *4CL-1* promoter.

Two separate assay systems were utilized to define *cis*-acting elements controlling *4CL-1* expression in response to stress. In the first, promoter deletions in the context of the entire *4CL-1* genomic clone were analyzed. These constructs were introduced into parsley protoplasts and stably transformed suspension-cultured cells were isolated. In each construct (Figure 1A), a 15 bp in-frame deletion in the first exon allowed discrimination between *4CL-1* mRNA transcribed from either the introduced template or the endogenous *4CL* genes. Total RNA from either individual transformants or pools of at least 5 individual transformants was analyzed by S1 nuclease protection using the end-labeled probe shown in Figure 1A. This probe was isolated from the wild-type *4CL-1* gene. Due to A-U richness in the 5′ untranslated leaders of *4CL-1* and *4CL-2* mRNAs, and some nucleotide mismatches between the two genes in this region, 'breathing' of DNA−RNA hybrids generates two protected fragments from the endogenous *4CL* genes (475 bp and 415 bp, Figure 1B). The combined signal intensity of these bands therefore is a measure of total *4CL* expression. These signals serve as internal controls for RNA amount loaded and for inducibility by fungal elicitor or light. RNA from the introduced *4CL-1* constructs protected a 213 bp fragment, since the location of the 15 bp deletion results in an S1 sensitive DNA loop in the probe at this point. Transformants

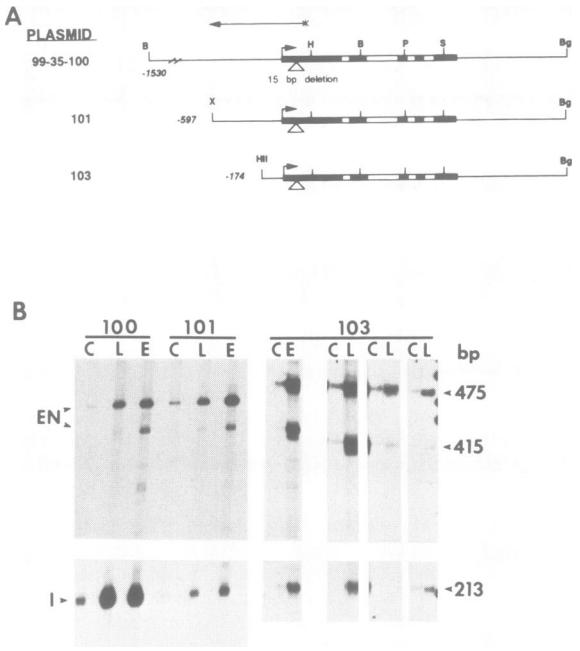

Fig. 1. Restriction maps of the *4CL-1* structural gene fused to *4CL-1* promoter fragments and their expression in transgenic parsley cell lines. (**A**) Structures of plasmid constructions stably introduced into parsley cells. Filled and open bars represent exons and introns, respectively, and lines 5′ and 3′ flanking sequences. The length of each 5′ flanking fragment is in base pairs immediately to the left of the construction. The transcription start site is indicated by an arrow. The location of a 15 bp deletion within the first exon of each construction is shown by a triangle, and the location of an end-labeled probe used for S1 nuclease protection assays is shown at the top of the Figure as a starred, horizontal arrow. All constructions are located in the polylinker of pRT99 (Toepfer *et al.*, 1988). Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; HII, *Hin*cII P, *Pst*I; S, *Sst*I; X, *Xho*I. (**B**) S1 nuclease protection assays of RNA expressed in clones stably transformed with the above plasmids. The protected 475 and 415 bp fragments from the endogenous genes (EN) and the 213 bp fragment from the introduced genes (I) are indicated with arrows. Pooled cell cultures of clones containing constructions 100 and 101 were either left untreated in the dark (C), irradiated with UV-containing white light (L), or treated with *Pmg* elicitor (E) before RNA extraction. Clones containing construction 103 were analyzed separately. A cell culture of pooled 103 clones was left untreated in the dark or treated with *Pmg* elicitor as above. Cell cultures of individual 103 clones were left in the dark or irradiated with UV-containing white light as above; three representative responses are shown.

containing a 1530 bp promoter (99-35-100) were highly responsive to both fungal elicitor and light (Figure 1B). A deletion derivative containing 597 bp of promoter (99-35-101) expressed quantitatively less *4CL-1* mRNA, but was still responsive to light and elicitor. A construct truncated to −174 bp (99-35-103) also retained both elicitor and light responsiveness, although expression was even further reduced (compare signals from endogenous with those from introduced genes). Clonal analysis from this construct revealed typical quantitative variability as shown in Figure 1B.

While the truncated constructions expressed less RNA, their inducibility remained similar to that of the endogenous genes. This was determined by scanning autoradiograms to quantify RNA amounts and by calculating the ratio of the induction of the introduced genes to the induction of the endogenous genes (relative induction value). A value of 1.0 would indicate equal induction of introduced and endogenous

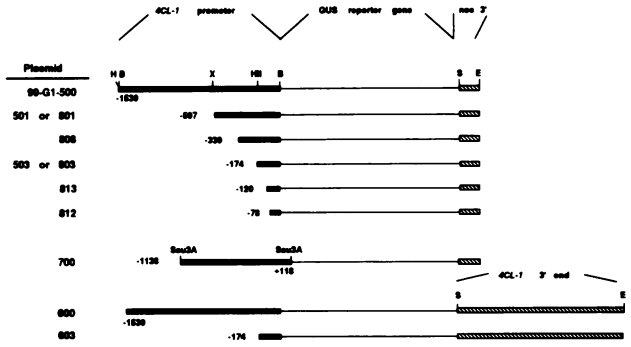

Fig. 2. Structure of *4CL-1-GUS* gene fusions tested for transient expression in parsley protoplasts or used to establish stably transformed parsley cell cultures. A restriction map of the *4CL-1* promoter region and the *GUS* gene with the nopaline synthase 3′ terminal region is shown above. Solid bars represent the promoter sequences fused to the *GUS* gene in each plasmid construction (base pair coordinates to the left are with respect to the *4CL-1* transcription start site) and striped bars represent either the nopaline synthase or *4CL-1* 3′ ends. All constructions are in pRT99 (Toepfer *et al.*, 1988). Plasmid 700 is a translational fusion; plasmids numbered 801 and higher are fusions of *4CL* sequences to a *GUS* gene derivative with a translation start codon surrounded by a eukaryotic consensus sequence; other transcription fusions (500 series and 600 series) lack this consensus sequence. Restriction sites: B, *Bam*HI E, *Eco*RI; H, *Hin*dIII; HII, *Hin*cII, S, *Sst*I; X, *Xho*I.

Table I. Expression of *4CL−GUS* transcriptional fusions in stably transformed parsley cell cultures

Independent clones from experiment	Plasmid	GUS activity (pM 4-MU/min × mg protein)		
		Control	Light	Elicitor
1	99-G1-500	4523	nt*	4411
	500	2684	nt	1641
	500	3827	nt	3666
	501	240	nt	142
	501	597	nt	682
	501	266	nt	265
	501	630	nt	553
2	500	4286	3001	3872
	501	859	760	895
	501	522	615	569
	503	80	60	94
	untransformed	42	nt	38
3	500	10 144	nt	10 790
	501	4086	nt	4565
	503	415	nt	405
	503	1351	nt	1342
	503	1192	nt	657
	503	788	nt	639
	503	1514	nt	843
	503	930	nt	1120
	untransformed	17	nt	25

*nt, not tested.

genes. In the experiments shown in Figure 1B, relative induction values for constructions 99-35-100, 101 and 103 after elicitor treatment were 1.1, 0.9 and 1.3, respectively; after light treatment the values were 2.5, 1.3 and 0.7 respectively. Scanning of autoradiograms from three separate experiments in which 99-35-101 expression was analyzed gave average values of 1.7 ± 0.8 and 1.4 ± 0.7 for elicitor and light treatments, respectively. Taken together, these data show that the inducibility of all constructions remains reasonably constant relative to that of the endogenous genes. The *cis*-acting elements required for inducibility thus appear to be located downstream of −174 bp, while sequences upstream of −174 bp affect quantity of *4CL-1* mRNA accumulation.

In the second assay system, these same three promoter fragments were fused transcriptionally to the β-glucuronidase (*uidA*) *GUS* reporter gene (Jefferson *et al.*, 1987) to generate plasmids 99-G1-500, -501, and -503 respectively (Figure 2). Individual clones from stably transformed suspension-cultured cells were analyzed separately for elicitor or light dependent induction of GUS activity. Surprisingly, GUS activity was in no case inducible (Table I). The general diminution of GUS activity with progressively shorter promoter fragments (compare 99-G1-500, -501, -503, Table I) is consistent with the S1 nuclease protection data presented in Figure 1. We investigated several possible artefacts which could potentially mask elicitor or light responsiveness of these constructs. First, we asked whether *4CL-1−GUS* mRNA amounts were inducible in these cultures. Figure 3 shows RNA 'slot blot' analysis after elicitor treatment of pools of clones whose GUS activity is given in Table I. In no case is *4CL-1−GUS* mRNA induced, although the endogenous *4CL* genes are highly and evenly induced in all three pools. A similar case exists for light induction (not shown).

Using a more rapid transient assay in parsley protoplasts, we investigated additional variables potentially responsible for the lack of *4CL* promoter response to elicitor or light when fused to the *GUS* reporter gene. Parsley protoplasts maintain differential response to elicitor and light (Dangl *et al.*, 1987) and this system has proven useful in definition of *cis*-acting elements from both light-regulated (Schulze-Lefert *et al.*, 1989a, 1989b; Block *et al.*, 1990) and elicitor-regulated (van de Löcht *et al.*, 1990) homologous promoters as well as heterologous promoters (Lipphardt *et al.*, 1988; Staiger *et al.*, 1990).

Table II shows typical transient expression data from several different sets of transcriptional and translational *4CL-1−GUS* fusions (see Figure 2 for constructions). No induction of GUS activity above a constitutive level was observed after either fungal elicitor or light treatment. This lack of induction was unrelated to the presence of either heterologous or homologous 3′ regions (experiment 1). Neither did DNA amount or the time between gene transfer and treatment have an effect on non-inducibility (experiment 1). Also, the 5′ untranslated region plays no apparent role (experiment 2). As observed in stably transformed parsley cells, 5′ truncation results in lower GUS activity, but no influence on non-inducibility was seen (experiment 2). Template topology also had no effect on lack of inducibility (not shown). Controls included a light responsive CHS−GUS fusion, a 35S−GUS fusion and the promotorless vector pRT99-GUS-JD.

The combination of S1 nuclease protection data shown in Figure 1 and the assembled *4CL-1−GUS* fusion data strongly argue that sequences downstream of +118 (the fusion point in 99-G1-700) are necessary for high level accumulation of *4CL-1* mRNA following elicitor or light treatment. To test this contention, we generated stably transformed parsley cells with vectors shown in Figure 4A.

Each contains the 597 bp *4CL-1* promoter fragment shown to mediate elicitor and light induction when in conjunction with the *4CL-1* genomic clone (Figures 1 and 4, construct 99-35-101). In constructs 99-35-200 and 99-35-201, this promoter drives the parsley *4CL-1* cDNA, also containing the 15 bp marker deletion described above. These two vectors differ in their 3′ segments. S1 nuclease protection analysis (Figure 4B) demonstrates that sequences in the *4CL* coding region render the 597 bp promoter fragment both elicitor and light responsive, and that *4CL* 3′ flanking DNA is not required. (Note that for 99-35-200, the apparent light induction is very low. This is due to underloading of that lane as can be seen by comparison of endogenous *4CL* mRNA amounts in the control and light treated samples for 99-35-200 and 99-35-201. In other experiments, light induction of 99-35-200 was obvious.)

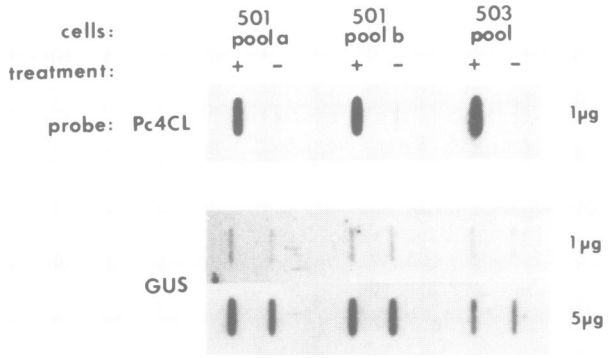

Fig. 3. RNA slot blot analysis of *4CL* and *GUS* transcript levels in transgenic parsley cell cultures. Two independent pools of clones transgenic for construction 501 and one such pool of clones transgenic for construction 503 were treated with elicitor (+) or left untreated (−). 1 μg of total RNA from each treatment was hybridized to a parsley *4CL* (Pc4CL) cDNA probe to detect accumulation of endogenous *4CL* transcripts. The same filter was stripped and subsequently hybridized to a *GUS* probe (upper *GUS* panel). To visualize *GUS*-specific transcripts better, 5 μg of each RNA on a separate blot was hybridized to *GUS* and the autoradiogram overexposed (lower *GUS* panel).

Stress responses in transgenic tobacco

To verify further the somewhat unconventional findings described above, we generated two lines of transgenic tobacco. One carried the entire parsley *4CL-1* genomic clone (construct 99-35-100 in Figure 1, but without the 15 bp deletion), the other the *4CL-1−GUS* fusion from 99-G1-801 (see Figure 2). We predicted that if tobacco cells responded similarly to parsley cells, expression from the first construct would be responsive to elicitor and light treatment, while expression from the latter would not. In addition, we were curious to see whether the *4CL-1* promoter was sufficient to direct the pattern of cell-type specific expression in leaves expected from *in situ* analyses in parsley (Schmelzer *et al.*, 1989) and predicted by analogy to expression patterns from the bean *PAL2* promoter in transgenic tobacco (Bevan *et al.*, 1989; Liang *et al.*, 1989b). It is important in this context to note that *Pmg* elicitor has been shown to induce phytoalexin biosynthesis in tobacco (Chappell and Noble, 1987). While phenylpropanoid enzymes are not used in the synthesis of this class of phytoalexins (sequiterpenoids), phenylpropanoid activity is probably required for lignification or deposition of wall-bound phenolics after pathogen attack.

In order to detect parsley *4CL-1* and endogenous tobacco *4CL* transcripts in the same transgenic plants, we relied on the fact that, while a potato *4CL* cDNA clone (St4CL, Fritzemeier *et al.*, 1988; Becker-André *et al.*, 1991) cross-hybridizes to tobacco *4CL*, this probe does not cross-hybridize to parsley *4CL-1*. Similarly, a *4CL-1* cDNA (Pc4CL) does not cross-hybridize to St4CL or tobacco *4CL* under the hybridization conditions used. RNA blot analysis (Figure 5) shows that the entire parsley *4CL-1* gene does, in fact, respond to stress treatment in transgenic tobacco. Following elicitor application, light treatment or wounding, mRNAs from both the endogenous tobacco *4CL* gene(s) and the introduced parsley *4CL-1* gene accumulate to high levels.

Table II. Transient expression of *4CL−GUS* fusions in parsley protoplasts

Experiment	Plasmid	μg DNA/10^6 protoplasts	Days after transfer[a]	GUS activity (pM 4-MU/min × mg protein)		
				Control	Light	Elicitor
1	99-Gl-500	20	3	660	nt[b]	630
	600	"	"	226	nt	160
	35S	"	"	800	nt	640
	600	20	0[c]	300	nt	320
	600	5	"	140	nt	160
2	500	20	0	56	53	57
	700	"	"	243	140	171
	801	"	"	179	99	127
	808	"	"	92	125	103
	803	"	"	97	104	74
	813	"	"	38	42	32
	812	"	"	18	15	15
	35S	"	"	278	362	nt
	CHS-GUS 041	"	"	42	1655	nt
	99-GUS-JD	"	"	9	20	14

[a]Treatments were applied on this day subsequent to DNA transfer.
[b]nt, not tested.
[c]For 0 day samples, treatment was begun immediately after DNA transfer.

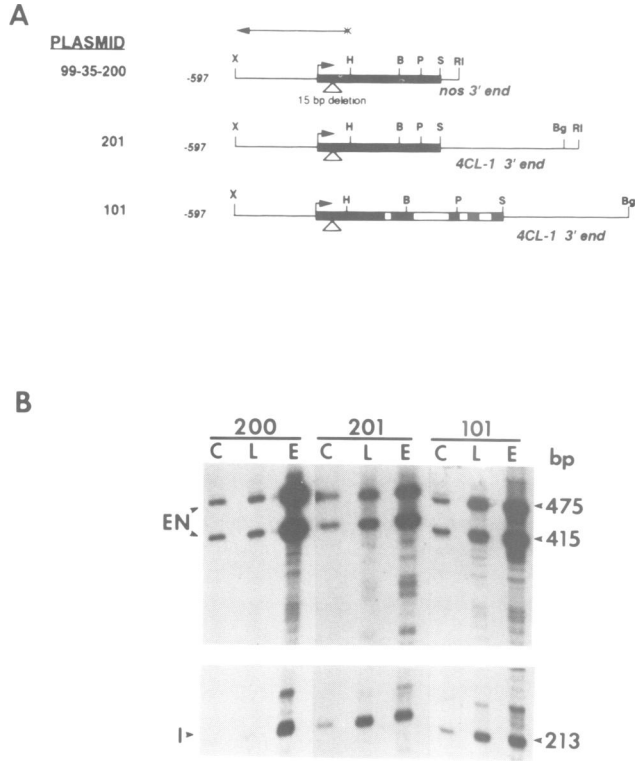

Fig. 4. Restriction maps and expression of transgenic parsley cell lines of *4CL-1* constructions with and without introns and the Pc4CL-1 3′ end. (**A**) Structures of plasmid constructions which were stably introduced into parsley cells. Filled and open bars represent exons and introns, respectively; the lines represent 5′ flanking sequences, and the origin of 3′ flanking DNA is indicated. The 5′ flanking fragment begins at an *Xho*I site at position −597 with respect to the transcription start site, which is indicated by an arrow in each construction. The location of a 15 bp deletion within the first exon of each construction is shown by a triangle, and the location of an end-labeled probe used for S1 nuclease protection assays is shown at the top of the figure as a starred, horizontal arrow. All constructions are in the polylinker of pRT99 (Toepfer *et al.*, 1988). Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; R1, *Eco*RI; S, *Sst*I; X, *Xho*I. (**B**) S1 nuclease protection assays of RNA expressed in pools of clones stably transformed with the above plasmids. The protected 475 and 415 bp fragments from the endogenous genes (EN) and the 213 bp fragment from the introduced genes (I) are indicated with arrows. Cell cultures containing each construction were either left untreated in the dark (C), irradiated with UV-containing white light (L), or treated with elicitor (E) before RNA extraction.

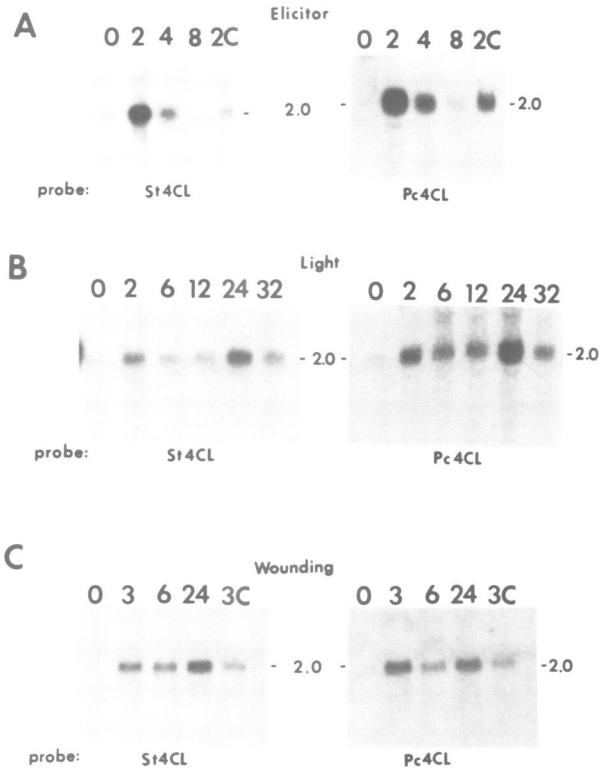

Fig. 5. Expression of tobacco *4CL* and Pc*4CL-1* genes in tobacco plants transgenic for *Pc4CL-1*. Total RNA from pooled tissue of 6 independent transgenic plants was subjected to RNA blot analysis. Duplicate blots were hybridized to a potato *4CL* cDNA (St4CL) probe for detection of endogenous tobacco *4CL* transcripts or to a parsley *4CL* cDNA (Pc4CL) probe for detection of transcripts from the introduced *4CL-1* gene (**A**) RNA from leaves 2, 4 and 8 h after treatment with *Pmg* elicitor solution and control leaves (2C) 2 h after treatment with water. (**B**) RNA from dark-grown plants irradiated with UV-containing white light for 0, 2, 6, 12, 24, and 32 h. (**C**) RNA from leaves 0, 3, 6 and 24 h after wounding and 3 h after detaching leaves and placing intact in water (3C). 10 μg total RNA was loaded in each lane.

We conclude that the parsley *4CL-1* gene is regulated by these stimuli in transgenic tobacco.

These data allowed us to address whether or not the *4CL-1* promoter was, firstly, sufficient to drive cell-type specific *GUS* expression, and secondly, insufficient for elicitor and light induction in transgenic plants, as predicted from data using parsley cells. Figure 6 shows that the 597 bp *4CL-1* promoter is sufficient to drive high levels of *GUS* expression in the developing xylem of a petiole from a young leaf (Figure 6A); vascular expression is also evident in leaves and stems (Figure 6D, E, F and G). We also observe *4CL-1*−*GUS* expression in roots, a variety of floral tissues and secondary xylem of stem (Hauffe *et al.*, 1991). These patterns are as predicted by analogy to *in situ* analyses of *4CL-1* expression in parsley (Schmelzer *et al.*, 1989). The only exception is the clear lack of *GUS* expression in epidermal cells, where light is thought to be a critical trigger in parsley. This promoter is insufficient, however, to generate *GUS* expression after either light treatment (Figure 6D,E) or elicitor application (Figure 6F,G). In the latter assay, however, we did detect GUS activity at the wound site. No elicitor-induced GUS accumulation is observed in the surrounding tissue, as would be expected if the *4CL-1* promoter were elicitor-responsive, by analogy to recent experiments in transgenic tobacco with the bean *CHS* promoters (Schmid *et al.*, 1990; Stermer *et al.*, 1990). As well, RNA blot analysis shows little or no induced accumulation of *GUS* mRNA (data not shown). Interestingly, Figures 6B and C show that the 597 bp *4CL-1* promoter is clearly wound responsive in epidermal cells of transgenic tobacco, in a manner similar to *4CL* in wounded parsley leaves (Schmelzer *et al.*, 1989). This demonstrates that the *4CL-1* promoter, although unresponsive to light in epidermal cell, can be activated in that cell type in transgenic tobacco. These data from transgenic tobacco confirm and extend the observations made in transformed parsley cells.

Discussion

We show in this paper that exonic sequences are required, in addition to homologous promoter sequences, for stimulated expression of the parsley *4CL-1* gene after fungal elicitor or light treatment. This conclusion is based on data

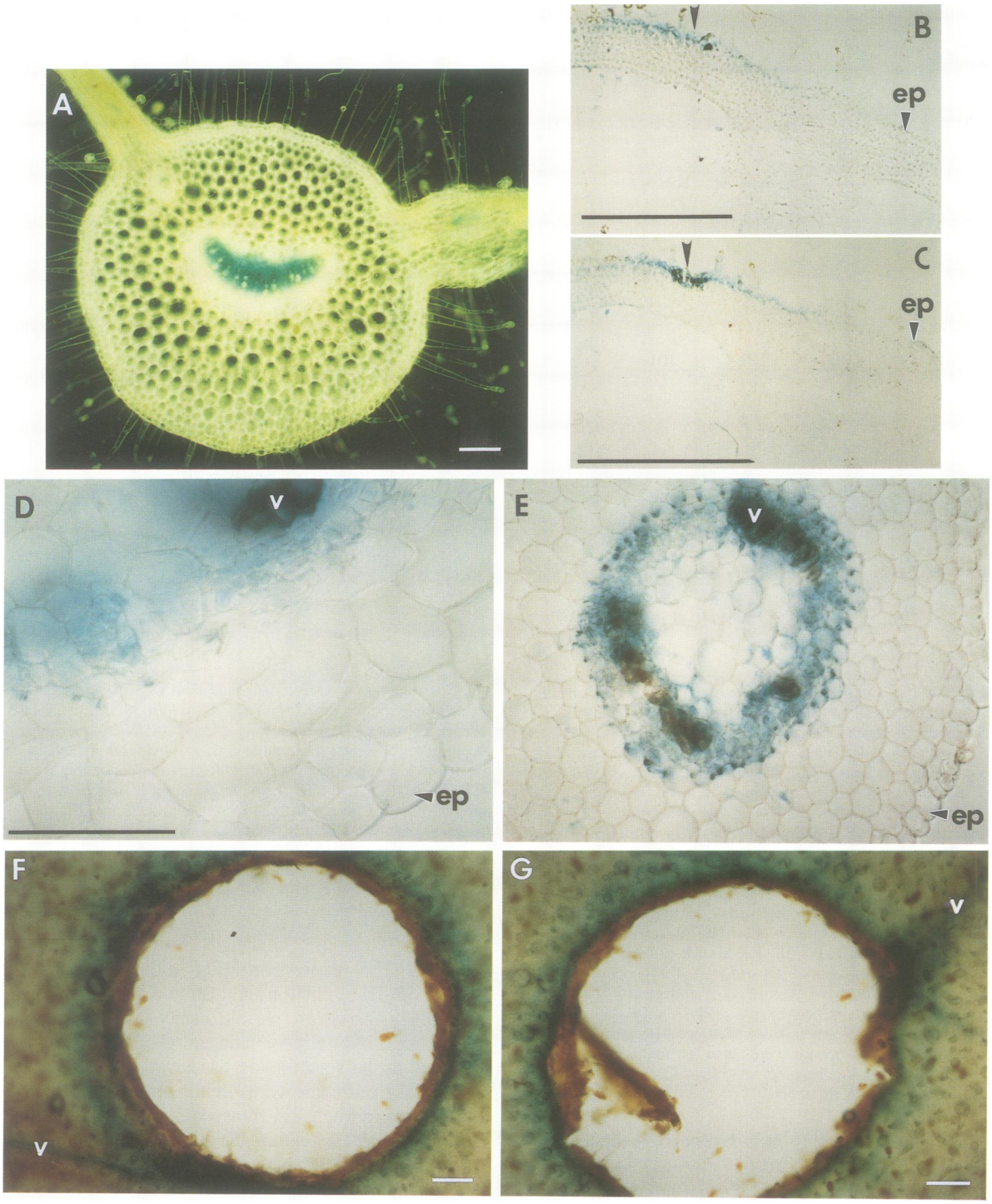

Fig. 6. Histochemical analysis of *GUS* gene expression in transgenic tobacco plants containing *4CL-1−GUS* fusion 801. Cells having GUS activity are recognized by indigo dye deposits after staining with X-GLUC. (**A**) Hand cross-section through an untreated young leaf of a light-grown plant. (**B−C**) Serial 20 μm sections through a wound site (arrow) on a young leaf. (**D**) Hand cross-section through the stem of a plant grown 2 weeks in dark. (**E**) Hand cross-section through the stem of a plant grown 2 weeks in dark then irradiated with UV-containing white light for 24 h. (**F**) Wound site of a leaf 24 h after application of 5 μl distilled water. (**G**) Wound site on the same leaf as in (F) 24 h after application of 5 μl of a *Pmg* elicitor solution. ep, epidermis; v, vascular tissue; bars equal 50 μm.

from both transformed suspension-cultured parsley cells and transgenic tobacco. We also show that a 597 bp *4CL-1* promoter fragment, although insufficient to render a reporter gene elicitor or light responsive, is capable of directing GUS expression in a tissue and cell-type specific manner. The data suggest that one set of *cis*-acting elements controls *4CL-1* response to developmental cues, while additional exonic sequences are needed for response to two important environmental stimuli. This arrangement of *cis*-linked controlling sequences is novel for phenylpropanoid and flavonoid genes analyzed to date. In each of those cases (Bevan *et al.*, 1989; Liang *et al.*, 1989b; Ohl *et al.*, 1990; Schmid *et al.*, 1990; van der Meer *et al.*, 1990), promoter sequences proved sufficient to regulate both developmentally and environmentally triggered reporter gene expression in heterologous transgenic plants.

How could exonic sequences act as *cis*-acting elements qualitatively controlling *4CL-1* promoter responsiveness? There are many reports of 5′ and 3′ untranslated exonic or intronic sequences from animal and viral genes which enhance steady-state mRNA accumulation (Moore *et al.*, 1985; Yang *et al.*, 1986; Coulombe *et al.*, 1988; Bornstein *et al.*, 1988; Basler *et al.*, 1989; Krauskopf *et al.*, 1990; Leong *et al.*, 1990). In plants, an intron from a maize alcohol-dehydrogenase gene affects steady state accumulation of mRNA (Callis *et al.*, 1987). As well, both the 3′ end of a potato proteinase inhibitor II gene, and the genomic 3′ region of a petunia ribulose bisphosphate carboxylase gene enhance accumulation of mRNA from heterologous promoters (An *et al.*, 1989; Dean *et al.*, 1989). Our results, in contrast, show clearly that exonic sequences, and not the *4CL-1* 5′ untranslated region, 3′ flanking DNA, or introns, are required for elicitor or light induction.

Although there is no precedent, to our knowledge, for enhancement of RNA polymerase II transcription rate by coding sequences, gene-internal binding sites are well documented for RNA polymerase III-associated transcription factors (Geiduschek and Tocchini-Valentini, 1988). It is clear that transcriptional activation is largely responsible for the *4CL-1* gene's response to elicitor and light, since both intron and exon probes have been used in nuclear run-on experiments (Chappell and Hahlbrock, 1984; Douglas *et al.*, 1987; Lois *et al.*, 1989). Thus, though unprecedented, it is possible that *4CL-1* exonic sequence(s) are acting as binding site(s) for RNA polymerase II associated transcription factors. An example potentially supporting this notion is the recent report of Elliot *et al.* (1989). They show that the transcribed portion of the intronless pea ferrodoxin I gene renders a heterologous promoter light responsive. In that case, it was not shown whether the homologous promoter was sufficient for tissue specific expression.

At this time, we cannot distinguish whether exonic sequences from the *4CL-1* gene act as transcriptional enhancers or mRNA stability elements. The latter mechanism is apparently involved in light-regulated expression of some chloroplast encoded genes (Gruissem, 1989). However, Douglas *et al.* (1987) directly compared the stimulation of transcription rate and *4CL* mRNA accumulation and found no evidence for post-transcriptional regulation. Thus, although it is difficult to postulate a control mechanism for the *4CL-1* gene based solely on mRNA stability, pre-mRNA stability could be involved.

Three sorts of experiments could be used to functionally identify potential exonic *cis*-acting elements. In one, all possible *Hin*dIII fragments from the *4CL-1* gene were subcloned into a position directly upstream of the −597 bp promoter−GUS fusion, and we asked if any could restore responsiveness to this promoter. In both stably transformed and transiently expressing parsley cells, no effect on background GUS level was observed (not shown). These experiments are hampered by disruption of possible spacing requirements between *cis*-acting elements, and are by no means definitive. An alternative would be to construct either in-frame deletions or 'linker-scanning' mutations through the entire *4CL-1* cDNA and look for loss of response in the context of the appropriate promoter. Another possible approach currently being pursued is to test for gain of inducibility when increasing lengths of *4CL-1* cDNA are fused translationally to the *GUS* reporter gene. Such approaches, however, are still unable to discriminate between *cis*-acting sequences acting as binding sites for either transcription or mRNA stabilizing factors.

Are *cis*-acting element(s) of the *4CL-1* promoter required, in addition to the enigmatic exonic sequence(s), to mediate *4CL-1* response to elicitor or light? To address this question, constructions were made in which the CaMV 35S promoter or a minimal 90 bp 35S promoter (both unresponsive to light or elicitor) was fused to the *4CL-1* cDNA. These constructions were unresponsive to either stimulus in transgenic tobacco plants (data not shown), demonstrating that *4CL-1* exonic sequences alone cannot confer responsiveness on an unresponsive heterologous promoter. These results are in contrast to those obtained with the pea ferrodoxin I gene (Elliot *et al.*, 1989) described above and suggest that *4CL-1* promoter sequences, in combination with exonic sequences, are required for inducible expression. Precedence for such interaction between the promoter and downstream elements is provided by Bornstein *et al.* (1988) who showed that interactions between *cis*-acting elements in the promoter and elements in the first intron of the human α-1(I) collagen gene are involved in regulating transcription of this gene.

Data were presented here showing that 174 bp of the *4CL-1* promoter, when fused to the *4CL-1* structural gene, is responsive (although weakly) to elicitor or light (Figure 1). Suggestions regarding *cis*-acting elements within this region which are required for responsiveness can be made based on sequence comparison to other phenylpropanoid and flavonoid gene promoters, and on *in vivo* footprinting from the parsley *PAL-1* and *4CL-1* genes (Lois *et al.*, 1989; Hauffe *et al.*, 1991).

Many guanine residues TATA proximal to −174 bp are constitutively footprinted in suspension-cultured parsley cells, and the pattern of *in vivo* methylation protection is only slightly modified after either elicitor or light treatment (Lois *et al.*, 1989; Hauffe *et al.*, 1991). An attractive candidate within this region is a sequence identified in the parsley *PAL-1* gene as the site of slight elicitor- and light-induced changes in putative protein−DNA contacts (Lois *et al.*, 1989). This sequence, 5′-CTCACCTACCA-3′, is found at −114 bp of the parsley *PAL-1* promoter. A 9 of 11 similarity, 5′-CTCACCAACCC-3′ is found at position −135 bp in the *4CL-1* promoter. Similar matches are found in the bean *PAL2* promoter, the bean *CHS15* promoter, and a soybean *CHS* promoter (Cramer *et al.*, 1989; Dron *et al.*, 1988; Wingender *et al.*, 1989, respectively). Interestingly, related sequences are found three times on an *Arabidopsis PAL* gene promoter, where functional analysis showed that 540 bp of promoter containing them is responsive to wounding, stress and light (Ohl *et al.*, 1990). A more localized mutagenic analysis of the TATA proximal 210 bp of the *4CL-1* promoter is underway, and should further define the role of these putative *cis*-acting elements.

Materials and methods

Recombinant DNA techniques

These were performed, with minor variations, according to Maniatis *et al.* (1982).

Plasmid constructions

A 15 bp deletion was created within the first exon of the *4CL-1* gene as follows: a cloned 2.7 kb *Bam*HI restriction fragment from the 5′ end of the *4CL-1* gene (containing 1.5 kb of upstream DNA and 1.2 kb of transcribed DNA; Douglas *et al.*, 1987; Lozoya *et al.*, 1988) was linearized at a unique *Sst*I site within the first exon, treated for 5 min on ice with

nuclease Bal31, the reaction stopped, plasmid ends filled with Klenow, and plasmids recircularized and sequenced across the deletion. The 5′ *Bam*HI site in a plasmid containing the desired deletion was deleted by filling in with Klenow, leaving an adjacent *Sal*I site in the polylinker intact to create plasmid 35-100. To construct plasmid 99-35-100, a 7.0 kb *Bgl*II *4CL-1* genomic restriction fragment containing the entire structural gene with 1.8 kb and 2.0 kb of flanking 5′ and 3′ DNA, respectively (Douglas *et al.*, 1987; Lozoya *et al.*, 1988), was cloned into the *Bam*HI site of pRT99 (Toepfer *et al.*, 1988), such that a polylinker *Sal*I site was adjacent to the 5′ end of the construct. A 3.0 kb *Sal*I−*Bam*HI from the 5′ end of this plasmid was replaced with the 2.7 kb *Sal*I−*Bam*HI restriction fragment from plasmid 35-100 to recreate a genomic copy of *4CL-1* with 1.5 kb of upstream DNA and a 15 bp deletion in the first exon. *Xho*I and *Hinc*II restriction sites within the 1.5 kb upstream region were used to create the deleted versions of 99-35-100 (99-35-101 and 103, Figure 3A).

Plasmid 99-35-201 was constructed by fusing an *Xho*I−*Cla*I fragment from plasmid 35-100 (containing 597 bp of upstream DNA and most of the first exon, including the 15 bp deletion) to the *Cla*I site of a *4CL-1* cDNA (Lozoya *et al.*, 1988), and replacing 200 bp of the 3′ untranslated portion of the cDNA with a 2 kb *Sst*I−*Eco*RI fragment of 3′ genomic flanking DNA which had previously been inserted into pRT99. In plasmid 99-35-200, 2 kb *Sst*I−*Eco*RI fragment was replaced by an *Sst*I−*Eco*RI fragment containing the nopaline synthase 3′ terminator.

Plasmids 99-G1-500 and 501 were constructed by cloning *4CL-1* promoter fragments as transcriptional fusions upstream of the *GUS* gene in pRT99-GUS-JD (Schulze-Lefert *et al.*, 1989a). A 1.5 kb promoter fragment was first isolated by Bal31 deletion of the 2.7 kb *Bam*HI fragment starting at the *Sst*I site 3′ to the transcription start site (Figure 2). This deletion derivative had a 3′ endpoint +17 with respect to the transcription start site and was cloned into pUC19 and pRT99-GUS-JD (to create 99-G1-500). 5′ deletion derivatives were created by standard procedures using Bal31 and upstream restriction sites. Plasmids 99-G1-801 through 813 were constructed by recloning the promoter fragments in pRT99−GUS-JD/Kozak, a derivative of pRT99-GUS-JD which contains a consensus eukaryotic ('Kozak') translation start site (Kozak, 1981; R.Jefferson, personal communication). To test the effect of the *4CL-1* 3′ end on GUS expression, an *Sst*I−*Eco*RI restriction fragment containing the nos termination region in pRT99-GUS-JD was replaced with a *4CL-1 Sst*I−*Eco*RI genomic restriction fragment containing 3′ untranslated sequences and 2 kb of flanking DNA (Lozoya *et al.*, 1988) in 99-G1-600 and 601. A translational fusion of 4CL-1 to GUS was constructed using a *Sau*3A fragment spanning nucleotides −1140 to +118 with respect to the transcription start site. This was inserted into the *Bam*HI site upstream of the *GUS* gene in a pRT99 version of pBI101.1 (Jefferson, 1987) to yield an in-frame fusion (construction 99-G1-700).

Protoplast preparation and transformation

Protoplasts were prepared from 5 day-old suspension cultured parsley cells (Dangl *et al.*, 1987) and transformation with supercoiled or linearized plasmid DNA performed as previously described (Lipphardt *et al.*, 1988; Schulze-Lefert *et al.*, 1989a). To test constructions for inducible expression, transformed protoplasts were divided; one sample was kept in the dark and one sample treated with UV-containing white light as described (Lipphardt *et al.*, 1988; Schulze-Lefert *et al.*, 1989a) or with *Pmg* elicitor (prepared from *Phytophthora megasperma* f. sp. *glycinea* as described, Ayers *et al.*, 1976; Kuhn *et al.*, 1984 and added at a concentration of 50 μg/ml). GUS activity in extracts of protoplasts was measured using the fluorometric assay described by Jefferson (1987) at several time points after the start of light elicitor, and control treatments.

Stably transformed parsley cell lines were obtained by plating protoplasts transformed as described above in liquid B5 media supplemented with 1 mg/l 2,4-D at a density of ~5 × 10^5/ml. After 2−3 weeks, extensive cell division had taken place and cells were placed on B5 media supplemented with 1 mg/l 2,4-D and 50 μg/ml kanamycin and solidified with 0.8% agar. Kanamycin-resistant clones which grew through the lawn of non-resistant cells were removed and maintained as calli or selective media. DNA blot analysis showed that 1−5 copies of the introduced DNA were present in the transformants. Suspension cultures were initiated by dispersing cells in liquid media on a rotary shaker. Suspension cultures of pools of clones transformed with the same construction were initiated by dispersing equal amounts of five or more individual clones into liquid media. After one week, 10 ml of the pool was transferred to 40 ml fresh media, grown for one week, and divided into 15 ml portions for dark, light and *Pmg* elicitor treatments (performed as described by Douglas *et al.*, 1987). Although pools were analyzed after two weeks of growth to ensure that extensive changes in their constitution did not take place, Southern blot profiles of the introduced genes in pools were essentially the same after 2 weeks and after 2 months in culture.

Tobacco transformation, elicitor, UV light and wound treatments

The *4CL−GUS* fusion in plasmid 99-G1-801 was recloned as an *Eco*RI−*Hin*dIII fragment, and the entire *4CL-1* gene as a *Bam*HI−*Bgl*II fragment into the *Agrobacterium* binary vector BIN19 (Bevan, 1984) and used to transform tobacco leaf discs by standard methods (Horsch *et al.*, 1985). Elicitor treatments were performed by excising fully expanded leaves of approximately the same age (4 months old) from greenhouse grown transformants and placing them in either distilled water or water containing 500 μg/ml *Pmg* elicitor under constant UV light. For histochemical analysis, small wounds were made in leaves left on plants. 5 μl water of *Pmg* elicitor solution at 50 or 500 μg/ml was added to the wound site. Light treatments were performed in two ways using axenically-grown F_1 seedlings. Seedlings ~2 weeks old were placed in constant darkness for 2 weeks, then removed and irradiated with constant UV-containing white light. Alternatively, constant UV-A irradiation was provided at a distance of 30 cm by a group of 4 Sylvania F20T12-BLB lamps which had maximum output at 350 nm to plants previously grown under white light in a regime of 16 h light/8 h dark. The *Pc4CL-1* gene was activated in a similar manner after either treatment. Wounding was performed by excising leaves of a similar age from 4 month old greenhouse-grown plants, cutting these into 1−2 mm strips and incubating on filter paper moistened with MS media in the absence of hormones. For 0 h time points, excised leaves were immediately frozen without further wounding; controls were also performed in which excised leaves were placed in water without further wounding. Leaves or seedlings from 6 independent transformants containing the entire *4CL-1* gene were pooled for RNA extraction following elicitor, light and wounding treatments.

Histochemical localization of GUS activity

Histochemical localization of GUS activity was performed as described by Jefferson (1987). Hand sections, tissue for paraffin embedding and sectioning, or whole mounts were taken from 4 month old greenhouse grown plants or axenically grown F_1 seedlings. Prior to staining, tissue was vacuum-infiltrated with 0.5% paraformaldehyde, 100 mM sodium phosphate (pH 7.0), 0.4 M sucrose for 1 h, then washed twice with 100 mM sodium phosphate (pH 7.0). Hand-sectioned tissue or tissue for whole mounts was stained for GUS activity 3−16 h using 5-bromo-4-chloro-3-indole glucuronide (X-GLUC, Research Organics, Cleveland, OH or Clontech, Palo Alto, CA) at 0.5 mg/ml in 10 mM sodium phosphate (pH 7.0). After staining, the tissue was cleared in ethanol (25−80%) and observed with a Zeiss Axioskope. Thicker tissue sections used for embedding were prefixed on ice and stained with X-GLUC as above for 16−18 h, then postfixed in 1% paraformaldehyde, 0.5% glutaraldehyde, 100 mM sodium phosphate (pH 7.0), and washed for 60 min with 100 mM sodium phosphate (pH 7.0), with buffer changes every 10 min. Tissue was dehydrated in a series of aqueous ethanol solutions (v/v) as follows: 25%, 40% and 55% for 20 min each; 70% overnight at 4°C; 80%, 90% and 95% for 20 min each at room temperature; 100% twice for 20 min. Subsequently, the tissue was infiltrated with tertiary butanol and paraplast as described by Schmelzer *et al.* (1989). Embedded tissue was sectioned at 20 μm thickness using a standard rotary microtome (Leitz, type 1212). Subsequent handling of tissue sections (relaxation, binding to microscope slides, solubilization of paraffin with xylene) was performed as described by Schmelzer *et al.* (1989).

Using these methods, endogenous GUS activity was not observed in any organs examined from untransformed tobacco plants.

RNA analysis

Total RNA was isolated from cultured parsley cells and tobacco plants by the method of Logemann *et al.* (1987). Northern blots were performed using Hybond (Amersham) according to the manufacturer's recommendations. Radioactive probes were prepared by random priming according to the manufacturer's specifications (Amersham). High stringency washes of hybridized blots were performed at 68°C in 0.2 × SSC, low stringency washes were at 68°C in 1 × SSC or 2 × SSC. To detect endogenous tobacco *4CL* transcripts, blots were hybridized to a potato *4CL* probe (St4CL, Fritzemeier *et al.*, 1988) and washed in 1 × SSC. Under these conditions, St4CL does not cross-hybridize to parsley *4CL*. Parsley *4CL* transcripts in transgenic plants were detected by hybridization of blots to a parsley *4CL* (Pc4CL-1, Lozoya *et al.*, 1988), and washing at high stringency. No hybridization of Pc4CL to RNA from untransformed tobacco plants was observed after low stringency washing (2 × SSC) of blots and prolonged exposure of autoradiograms.

An end-labeled *Xho*I−*Hin*dIII probe spanning the transcription start site and the introduced 15 bp deletion was used in S1 nuclease protection assays. Labeling was achieved by digestion of a cloned *Hin*dIII−*Bam*HI fragment with *Hin*dIII, end-labeling with T4 polynucleotide kinase, digestion with *Xho*I, and gel purification of the end labelled *Xho*I−*Hin*dIII fragment. 15 μg total RNA was mixed with 100 000−200 000 c.p.m. (about 50 ng) probe

of 30 μl hybridization buffer (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA and 80% formamide), denatured 10 min at 65°C, and hybridized 16 h at 50°C. 300 μl ice-cold S1 digestion buffer (30 mM sodium acetate, pH 4.6, 200 mM NaCl, 4.5 mM $ZnSO_4$) containing 200 units/ml S1 nuclease (Boehringer Mannheim) was added and the reaction was incubated 30 min at 20°C. The reaction was stopped by the addition of 30 μl 0.5 M EDTA, nucleic acids precipitated by addition of an equal volume of isopropanol, and run on a 6% sequencing gel. Autoradiograms were scanned with an LKB laser densitometer.

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References

An,G., Mitra,A., Choi,H.K., Costa,M.A., An,K., Thornburg,R.W. and Ryan,C.A. (1989) *The Plant Cell*, **1**, 115–122.

Ayers,A.R., Ebel,J., Valent,B. and Albersheim,P. (1976) *Plant Physiol.*, **57**, 760–765.

Basler,K., Siegrist,P. and Hafen,E. (1989) *EMBO J.*, **8**, 2381–2386.

Becker-Andre,M., Schulze-Lefert,P. and Hahlbrock,K. (1991) *J. Biol. Chem.*, in press.

Bevan,M.D. (1984) *Nucleic Acids Res.*, **12**, 8711–8721.

Bevan,M., Shufflebottom,D., Edwards,K., Jefferson,R. and Schuch,W. (1989) *EMBO J.*, **8**, 1899–1906.

Block,A., Dangl,J.L., Hahlbrock,K. and Schulze-Lefert,P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5387–5391.

Bornstein,P., McKay,J., Liska,D.J., Apone,S. and Devarayalu,S. (1988) *Mol. Cell. Biol.*, **8**, 4851–4857.

Callis,J., Fromm,M. and Walbot,V. (1987) *Genes Dev.*, **1**, 1183–1200.

Chappell,J. and Hahlbrock,K. (1984) *Nature*, **311**, 76–78.

Chappell,J. and Noble,R. (1987) *Plant Physiol.*, **85**, 469–473.

Collinge,D.B. and Slusarenko,A.J. (1987) *Plant Mol. Biol.*, **9**, 389–410.

Coulombe,B., Ponton,A., Daigneault,L., Williams,B.R.G. and Skup,D. (1988) *Mol. Cell. Biol.*, **8**, 3227–3234.

Cramer,C.L., Ryder,T.B., Bell,J.N. and Lamb,C.J. (1985) *Science*, **227**, 1240–1243.

Cramer,C.L., Edwards,K., Dron,M., Liang,X., Dildine,S.L., Bolwell,G.P., Dixon,R.A., Lamb,C.J. and Schuch,W. (1989) *Plant Mol. Biol.*, **12**, 367–383.

Dangl,J.L. (1991) In Boller,T. and Meins,F. (eds), *Plant Gene Research*, Springer-Verlag, Berlin. vol. 8, in press.

Dangl,J.L., Hauffe,K.D., Lipphardt,S., Hahlbrock,K. and Scheel,D. (1987) *EMBO J.*, **6**, 2551–2556.

Dena,C., Favreau,C.M., Bond-Nutter,D., Bedbrook,J. and Dunsmuir,P. (1989) *The Plant Cell*, **1**, 201–208.

Dixon,R.A. and Harrison,M.S. (1990) *Adv. Genet.*, **28**, 165–234.

Douglas,C., Hoffmann,H., Schulz,W., and Hahlbrock,K. (1987) *EMBO J.*, **6**, 1189–1195.

Dron,M., Clouse,S.D., Lawton,M.A., Dixon,R.A. and Lamb,C.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6738–6742.

Ebel,J. (1986) *Annu. Rev. Phytopathol.*, **24**, 235–264.

Elliot,R.C., Dickey,L.F., White,M.J. and Thompson,W.F. (1989) *The Plant Cell*, **1**, 691–698.

Fritzemeier,K.-H., Cretin,C., Kombrink,E., Rohwer,F., Taylor,J., Scheel,D. and Hahlbrock,K. (1987) *Plant Physiol.*, **85**, 34–41.

Geiduschek,E.P. and Tocchini-Valentini,G.P. (1988) *Annu. Rev. Biochem.*, **57**, 873–914.

Gruissem,W. (1989) *Cell*, **56**, 161–170.

Hahlbrock,K., Kreuzaler,F., Ragg,H., Fautz,E. and Kuhn,D.N. (1982) In Jaenicke,L. (ed.), *Biochemistry of Differentiation and Morphogenesis*. Springer-Verlag, Berlin, pp. 34–43.

Hahlbrock,K. and Scheel,D. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 347–369.

Hauffe,K.D., Paszkowski,U., Schulze-Lefert,P., Hahlbrock,K., Dangl.,J.L. and Douglas,C.J. (1991) *Plant Cell*, **3**, in press.

Horsch,R.B., Fry,J.E., Hoffmann,N.L., Eichhotz,D., Rogers,S.G. and Fraley,R.T. (1985) *Science*, **227**, 1229–1231.

Jahnen,W. and Hahlbrock,K. (1988) *Planta*, **173**, 197–204.

Jefferson,R.A. (1987) *Plant Mol. Biol. Rep.*, **5**, 387–405.

Jefferson,R.A., Kavanagh,T. and Bevan,M.W. (1987) *EMBO J.*, **6**, 3901–3907.

Kaulen,H., Schell,J. and Kreuzaler,F. (1986) *EMBO J.*, **5**, 1–8.

Kozak,M. (1981) *Nucleic Acids. Res.*, **9**, 5233–5252.

Krauskopf,A,. Resnekov,O. and Aloni,Y. (1990) *J. Virol.*, **64**, 354–360.

Kuhn,D.N., Chappell,J., Boudet,A. and Hahlbrock,K. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1102–1106.

Lamb,C.J., Lawton,M.A., Dron,M., Dixon,M. and Dixon,R.A. (1989) *Cell*, **56**, 215–224.

Lawton,M.A. and Lamb,C.J. (1987) *Mol. Cell. Biol.*, **7**, 335–341.

Lawton,M.A., Clouse,S.D. and Lamb,C.J. (1990) *Plant Cell Rep.*, **8**, 561–564.

Leong,K., Lee,W. and Berk,A.J. (1990) *J. Virol.*, **64**, 51–60.

Liang,X., Dron,M., Cramer,C.L., Dixon,R.A. and Lamb,C.J. (1989a) *J. Biol. Chem.*, **264**, 14486–14492.

Liang,X., Dron,M., Schmid,J., Dixon,R.A. and Lamb,C.J. (1989b) *Proc. Natl. Acad. Sci. USA*, **86**, 9284–9288.

Lipphardt,S., Brettschneider,R., Kreuzaler,F., Schell,J. and Dangl,J.L. (1988) *EMBO J.*, **7**, 4027–4033.

Logemann,J., Schell,J. and Willmitzer,L. (1987) *Anal. Biochem.*, **163**, 16–20.

Lois,R., Dietrich,A. and Hahlbrock,K. (1989) *EMBO J.*, **8**, 1641–1648.

Lozoya,E., Hoffmann,H., Douglas,C.J., Schulz,W., Scheel,D. and Hahlbrock,K. (1987) *Eur. J. Biochem.*, **176**, 661–667.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning—A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mol,J.N.M., Stuitje,A.R., Gerats,A.M.G. and Koes,R.E. (1988) *Plant Mol. Biol. Rep.*, **6**, 274–278.

Moore,D.D., Marks,A.R., Buckley,D.I., Kapler,G., Payvar,F. and Goodman,H.M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 699–702.

Ohl,S., Hedrick,S., Choury,J. and Lamb,C.J. (1990) *The Plant Cell*, **2**, 337–348.

Scheel,D., Hauffe,K.D., Jahnen,W. and Hahlbrock,K. (1986) In Lugtenberg,B. (ed.), *Recognition in Microbe–Plant Symbiotic and Pathogenic Interactions*. Springer-Verlag, Berlin, pp. 325–331.

Schmelzer,E., Jahnen,W. and Hahlbrock,K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2989–2993.

Schmelzer,E., Krüger-Lebus,S. and Hahlbrock,K. (1989) *The Plant Cell*, **1**, 993–1001.

Schmid,J., Doerner,P.W., Clouse,S.D., Dixon,R.A. and Lamb,C.J. (1990) *The Plant Cell*, **2**, 619–631.

Schulze-Lefert,P., Dangl,J.L., Becker-André,M., Hahlbrock,K. and Schulz,W. (1989a) *EMBO J.*, **8**, 651–657.

Schulze-Lefert,P., Becker-André,M., Schulz,W., Hahlbrock,K. and Dangl,J.L. (1989b) *The Plant cell*, **1**, 707–714.

Staiger,D., Kaulen,H. and Schell,J. (1990) *Plant Physiol.*, **93**, 1347–1353.

Stermer,B.A., Schmid,J., Lamb,C.J. and Dixon,R.A. (1990) *Mol. Plant-Mic. Int.*, **3**, 381–388.

Tietjen,K.G., Hunkler,D. and Matern,U. (1983) *Eur. J. Biochem.*, **131**, 401–407.

Toepfer,R., Schell,J. and Steinbiss,H.-H. (1988) *Nucleic Acids Res.*, **16**, 8725.

van de Löcht,U., Meier,I., Hahlbrock,K. and Somssich,I. (1990) *EMBO J.*, **9**, 2945–2950.

van der Meer,I., Spelt,C.E., Mol,J.N.M. and Stuitje,A.R. (1990) *Plant Mol. Biol.*, **15**, 95–109.

Wingender,R., Rohrig,H., Horicke,C., Wing,D. and Schell,J. (1989) *Mol. Gen. Genet.*, **218**, 315–322.

Yang,J.-Q., Remmers,E.F. and Marcu,K.B. (1986) *EMBO J.*, **5**, 3553–3562.

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