UV-inducible transient expression in parsley protoplasts identifies regulatory *cis*-elements of a chimeric *Antirrhinum majus* chalcone synthase gene

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It was shown previously that parsley protoplasts retain differential responsiveness to external stimuli, e.g. UV light. This opened the way for the development of transient expression assays to identify DNA sequences acting as regulatory *cis*-elements in the transcriptional activation of UV-inducible plant genes. The chalcone synthase (*chs*) gene of *Antirrhinum majus* (Snapdragon) is inducible by visible and by UV light. We demonstrate that the kinetics of light induction of a chimeric *A. majus chs-nptII* gene observed during transient expression in the parsley protoplasts reflect the kinetics of UV-induced CHS expression in *A. majus* seedlings *in vivo*. We define three regulatory sequence regions, each differing qualitatively and quantitatively in their effects on gene expression. Immediately upstream of the TATA box (–34) a sequence, with coordinates –39 to –197, functions as an orientation independent UV-light responsive element. The complete 1.1 kb promoter or three tandem copies of this element are capable of rendering a heterologous minimal promoter UV responsive. The next upstream region (–197 to –357) contains sequences that do not by themselves cause UV-induced expression, but specifically potentiate the level of UV-induced expression when combined with the TATA-box proximal UV-responsive element. A third element (–661 to –564) has the properties of a general enhancer since it increases the level of both uninduced and UV-induced expression.

Key words: chalcone synthase/regulated transient expression/UV light/parsley protoplasts/*cis*-acting regulatory elements/chimeric gene

Introduction

Chalcone synthase (CHS) is the key enzyme in the biosynthesis of flavonoids in plants. Various classes of flavonoids function as flower pigments, UV-stress protectants and phytoalexins. The functional complexity suggests that the regulation of *chs* gene expression is likely to be multifaceted (for review see Dangel *et al.*, 1988). The *chs* gene of *Antirrhinum majus* was recently isolated and its structure analysed (Sommer and Saedler, 1986). Since there is genetic evidence that the genome of *A. majus* has only a single *chs* gene, it must contain all the regulatory sequences

essential for its complex developmental and inducible regulation.

Two approaches have thus far been used to identify the *cis*-acting regulatory sequences of the *A. majus chs* gene. Kaulen *et al.* (1986) constructed a chimeric *chs-nptII* gene (NPTII, neomycin phosphotransferase II) and studied its expression and induction in transgenic tobacco plants. They showed that a 1.1 kb 5' upstream sequence of the *A. majus chs* gene contained all the necessary information to drive UV-induced expression of *chs-nptII* chimeric genes in transgenic tobacco seedlings. By deletion of parts of the 1.1 kb 5' upstream region, subregions were identified which quantitatively influenced gene expression in teratoma calli. Preliminary data indicated that sequences located downstream of –357 are essential for the response of the *A. majus chs* promoter to UV light and that sequences located upstream of –357 are involved in maximal gene expression (Kaulen, 1986).

Sommer *et al.* (1988) studied the influence on gene expression of well defined transposon-induced mutations in the promoter region of the resident *A. majus chs* gene. They found that the loss of one of two repeated hexamers (TACCAT, –43 and –55) located upstream of the TATA box (–34) diminished the constitutive level of CHS expression in white light grown plants.

Since Dangel *et al.* (1987) recently showed that parsley protoplasts retain differential responsiveness to UV light and to fungal elicitor, we developed an UV-light inducible transient expression system in parsley protoplasts. This system allows a convenient and rapid assay for promoter analysis and is not subject to potential 'position effects' resulting from insertion of foreign genes at different locations in the plant genome (Jones *et al.*, 1985; An, 1986; Paulsen *et al.*, 1986; Schell *et al.*, 1987; Gidoni *et al.*, 1988). In this paper, we report the use of the transient system to study the *cis*-elements involved in expression and UV induction of the chimeric *A. majus chs-nptII* gene and we compare the transient induction kinetics to results obtained with *A. majus* seedlings *in vivo*. These results with a heterologous promoter in parsley protoplasts demonstrate the potential utility of such analyses in properly responding cell systems.

Results

UV induction of endogenous *A. majus* CHS *in vivo*

In order to interpret more appropriately the expression and UV induction data of chimeric *chs-nptII* genes in parsley protoplasts, we first analysed the expression of the endogenous *A. majus chs* gene *in vivo*. Etiolated seedlings and green seedlings (2–3 weeks old) grown under a UV-protecting plexiglass shield (UV-free) were irradiated with continuous white light (Phillips TL20W18 lamps including wavelengths of 300–400 nm, referred to throughout as UV light). At the indicated time points, samples were taken and

Fig. 1. Detection of CHS protein in *A. majus* seedlings prior to and after UV irradiation. UV-protected green (A) and etiolated (B) seedlings were treated with continuous UV irradiation for the indicated time. Crude extracts of 20 mg fresh weight of seedlings were analysed via Western blots with CHS antiserum. In white-light adult grown *A. majus* plants (op), CHS protein is present in leaf tissue without UV induction. In green tissue a cross-contaminating protein of 66 kd in size is seen.

protein extracts were analysed by Western blotting. In both UV-free (Figure 1a) and etiolated (Figure 1b) seedlings, CHS protein was present prior to UV induction. The amount of CHS protein increased after 12–15 h of continuous irradiation. As well, total RNA from the same seedlings was extracted and analysed by Northern blotting (Figure 2). In etiolated seedlings (Figure 2b) CHS mRNA amount increased after 3 h of UV irradiation and showed a sharp optimum after 12 h of UV-induction. After 24 h of continuous treatment the amount of message decreased, although not to pre-induction levels. The accumulation of CHS mRNA in 'UV-free' grown seedlings showed less overall induction and peaked between 12 and 15 h (Figure 2a). The level of message decreased rapidly, but some CHS mRNA remained after 24 h of induction.

The small amount of CHS mRNA in uninduced etiolated seedlings was detectable after application of 50–60 μ g of total RNA to the assay (data not shown). The induction factor of CHS mRNA determined by laser densitometric scanning was ~10- to 20-fold in etiolated seedlings, and clearly lower in 'UV-free' seedlings which had been exposed to visible light during their growth period. We also detected CHS mRNA in adult *A. majus* plants grown in visible light (Figure 2). These data indicate that the expression of the *chs* gene is detected in all investigated stages of *A. majus* plants and that it is inducible by UV irradiation. We note, however, that prepriming with visible light affects both the time course and magnitude of CHS mRNA accumulation. These data are consistent with the demonstrated involvement of three photoreceptors (blue, red and UV) in *chs* gene activation (Bruns *et al.*, 1987; Ohl *et al.*, 1988; Schmelzer *et al.*, 1988) and subsequent flavonoid accumulation (Duell-Pfaff and Wellman, 1982; Oelmüller and Mohr, 1985).

Kinetics of UV-induced transient expression of chimeric *chs* – *nptII* genes in parsley protoplasts

Previous work indicated that preparation of parsley protoplasts does not harm the expression of enzymes of flavonoid biosynthesis (Dangl *et al.*, 1987). By Western blot

Fig. 2. Time course of UV induction of CHS mRNA in *A. majus* seedlings. CHS mRNA accumulation from (a) UV-free seedlings and (b) etiolated seedlings was measured using RNA blot hybridization. From the same seedlings used in Figure 1, total RNA was extracted and a Northern blot with 20 μ g of RNA hybridized with an *A. majus* CHS cDNA (Sommer and Saedler, 1986). In white-light grown old plants (op) CHS mRNA is constitutively present. The positive control (+) is total RNA extracted from an 8-h UV-induced parsley suspension culture.

analysis of protoplast extracts, we showed that neither polyethyl glycol (PEG) treatment nor DNA introduction of up to 50 μ g of DNA containing CHS promoter sequences diminished the UV induction of the resident parsley CHS (Lipphardt, 1988). Thus the transformation procedure does not affect UV inducibility and putative *trans*-acting factors are not titrated by the addition of an excess amount of potential template.

We optimized the conditions for UV inducible transient expression using the chimeric *chs* – *nptII* promoter fragment of construct 714 (see Figure 4). This 1.1 kb CHS promoter fragment was shown to be UV-light responsive in transgenic tobacco seedlings (Kaulen *et al.*, 1986). We observed the highest levels of light induced transient NPTII activity in protoplasts by initiating UV-light treatment immediately after DNA transfer. A time course of NPTII expression in this protocol is shown in Figure 3. Transformation efficiency in these experiments is controlled as described in Materials and methods. Low level constitutive NPTII activity in dark control protoplasts increases slowly during the first 24 h after DNA transfer (open circles). Light induced NPTII activity is first clearly visible 6 h after DNA transfer and onset of light treatment. Induced activity climbs rapidly during the first 15 h, and then levels off (closed circles). Thus, highest induction above the background level (triangles) occurs at ~15 h after gene transfer and immediate onset of light treatment.

The ascending kinetics and overall magnitude of UV-light induced NPTII activity from the exogenous DNA template correlate well with mRNA accumulation in etiolated *A. majus* seedlings as described above. We therefore consider the UV-light responsiveness of the *A. majus* CHS promoter in parsley protoplasts to reliably reflect its *in vivo* mode(s) of UV-light activation.

Construction of CHS – NPTII promoter mutants

The *A. majus* CHS promoter constructs tested for light

Fig. 3. Time course of UV induction of a *chs-nptII* chimeric gene by transient expression in parsley protoplasts. A transient expression experiment was performed with 12×10^6 protoplasts. Light induction of half of them started directly after transformation. At indicated time points, aliquots of 1×10^6 UV-irradiated or 1×10^6 dark-control protoplasts were frozen and later analysed by NPTII assay. After autoradiography, the radioactivity of each NPTII spot was counted in scintillation liquid and a curve drawn. Open dots show the slow increase of constitutive NPTII expression in the dark, while filled dots represent the UV-induced NPTII expression. Open triangles demonstrate the relative light induced expression (induction factor) with time. Each type of data are plotted on the same numerical scale.

responsiveness in the heterologous parsley protoplast system are shown in Figure 4. They include several constructs whose activity in transgenic tobacco has been chronicled (Kaulen, 1986; Kaulen *et al.*, 1986) as well as further constructs based on sequence characteristics indicative of potential *cis*-regulatory elements. All constructs have identical translation fusions to the *nptII* coding sequence, and all have a 3' end derived from the parsley CHS genomic clone (Kreuzaler *et al.*, 1983; Reimold *et al.*, 1983; Herrmann *et al.*, 1988). A *KpnI* fragment (–39 to –197) containing the two distinct repeats (–55) TACCAT acttgg TACCAT (–38) was shown to affect overall CHS mRNA levels in white light grown *A.majus* (Sommer *et al.*, 1988). The minimal representation of this fragment is construct $\Delta 5$. An extremely AT-rich region at approximately –300 shares features thought to be involved in the regulation of the gene encoding the small subunit (SSU) of ribulose biphosphate decarboxylase (Green *et al.*, 1987; Fluhr *et al.*, 1986). This sequence is contained in a fragment with coordinates –197 to –357. Finally, Kaulen *et al.* (1986) noticed the presence

Fig. 4. Map of *chs-nptII* promoter constructs. The graph shows 5' region of the complete 1.1 kb *chs-nptII* gene up to the junction with the *nptII* gene. Important restriction sites are indicated and important *cis*-elements described in the text are differentially shaded. The completely filled section marks the promoter region maintained in all constructs except the heterologous NOS minimal promoter, which is dotted. Cloning procedures are detailed in Materials and methods. 5 Nos carries three *KpnI*–*KpnI* fragments in tandem.

of an enhancer core sequence flanked by two 47-bp direct repeats (–661 to –564). Promoters containing these fragments, in various combinations, orientations or with a heterologous TATA box, were constructed using standard techniques.

Fig. 5. Identification of CHS UV-responsive *cis*-elements by transient expression. Protoplasts (2×10^6) were transformed with the indicated promoter constructs, all of which contained the *cis*-element from positions -39 to -197 . Half of the protoplasts were UV-irradiated for 15 h directly after the transformation and half kept in the dark. Autoradiogram of the NPTII assay (a) and a block diagram of counts incorporated in kanamycin (b) are shown. Induction factors refer to either the NPTII assay presented in (a) or to the average of five independent experiments (Lipphardt, 1988).

Identification of light responsive and enhancing *cis*-elements from the *A.majus* CHS promoter

A representative NPTII assay, including all promoter constructs which maintained light responsiveness in the parsley protoplast transient expression system, is shown in Figure 5a. The full 1.1 kb CHS promoter fragment of construct 714 directed NPTII expression which, though clearly present in the dark, was strongly induced after light treatment. A deletion of -357 ($\Delta 2$) does not affect the light responsiveness of the *A.majus* CHS promoter, but rather lowers overall levels in both dark control and light activated protoplasts (see Figure 5b). Further truncation to -197 generates the shortest promoter fragment tested ($\Delta 5$) which

retains UV-light responsiveness. This promoter fragment, however, is severely impaired in both light induced and dark control expression levels. A control construct consisting only of 39 bp upstream of the transcriptional start site ($\Delta 4$) is unable to direct NPTII expression (data not shown).

These data suggest that the fragment from -37 to -197 contains sequence(s) necessary for light activation of the *A.majus* CHS promoter (see also below). In addition, upstream fragments have two notably different quantitative effects. First, sequences from -197 to -357 seem to function as a light dependent enhancer. This fragment is not demonstrably responsible for CHS expression in the dark (compare 714 to $\Delta 2$), but it accentuates light activated expression (compare $\Delta 2$ to $\Delta 5$). Calculation of induction factors from five independent experiments suggests that this element directs the majority of high level UV-light responses. Second, a light independent enhancer resides between -564 and -661 . This element is somewhat position independent (see $\Delta 8$).

To confirm the necessity of sequences from -39 to -197 in light responsiveness, deletion mutants lacking this region were constructed ($\Delta 1$, $\Delta 3$ and $\Delta 6$; Figure 4). Each of these promoters retained fragments containing either or both upstream quantitative elements. Figure 6 shows NPTII activity in parsley protoplasts analysed three days after gene transfer. This was necessary to allow accumulation of NPTII activity from weakly expressing mutants. Note, however, that the light inducible constructs 714 and $\Delta 5$, included in this experiment as controls, behave qualitatively the same as in the normal protocol with immediate light treatment. Figure 6 shows that all promoter mutants lacking the sequences between -39 and -197 are non-light responsive. Thus, construct $\Delta 5$ contains the minimal light activated promoter thus far constructed and sequences defined by $\Delta 5$ are necessary for light responsiveness.

The data in Figure 6 also show the properties of the enhancer-like upstream fragments. First, as outlined above, the presence of sequences from -564 to -661 increases constitutive NPTII activity and can be defined as a general enhancer. The fragment spanning -197 to -357 is able to direct only very low non-light responsive levels of NPTII expression alone ($\Delta 6$), but apparently works in concert with the general enhancer (compare $\Delta 1$ and $\Delta 3$). Recall that this fragment influences the magnitude of light dependent expression when present together with the minimal light responsive element of -39 to -197 ($\Delta 2$, Figure 5, compare to $\Delta 5$).

The light responsive element of the *A.majus* CHS promoter between -39 and -197 also functions in reverse orientation (Figure 7a). When this fragment is internally inverted in the context of either the full 1.1 kb promoter (714R) or in the presence of only the general enhancer ($\Delta 7R$), UV-light response is maintained. Finally, we show that both the full 1.1 kb promoter fragment and the light responsive element from -39 to -197 (albeit as a triplet) can confer light regulation onto a heterologous minimal promoter (Figure 7b).

Discussion

This paper presents the delineation of three functionally distinct regulatory units from the UV-light inducible *A.majus* *chs* gene promoter. These were defined in a heterologous,

Fig. 6. *chs-ntpII* constructs lacking the fragment -39 to -197 are inducible. Parsley protoplasts (2×10^6) were transformed with the indicated *chs-ntpII* constructs (separated into equal aliquots) and kept in the dark for 2 days. After subsequent UV-light treatment for 15 h, samples and dark controls were analysed by NPTII assay. The low, but detectable and reproducible, repression of constitutively expressing constructs observed after light treatment is also observed in the case of a co-transferred CaMV 35S-CAT reporter gene (not shown).

Fig. 7. UV-responsive elements function are orientation and TATA box independent. (a) The orientation of the *KpnI* fragment at -197 to -39 was internal inverted in the 1.1 kb promoter 714 to give construct 714R and in construct $\Delta 7$ to give $\Delta 7R$. (b) The *A. majus* CHS promoter from 714 or three tandem copies of the UV-responsive *cis*-element from five were fused to a *nos* minimal promoter. The protoplasts transformed with 714 and 714R were induced 10 h after transformation. The vertical line indicates that two individual experiments are shown.

light responsive protoplast system (Dangl *et al.*, 1987). Our results rest largely on the fidelity of induced transient expression from the *A. majus* CHS promoter in parsley protoplasts. To address this, we show that the kinetics of light regulated CHS mRNA accumulation in etiolated *A. majus* seedlings is recapitulated by induction of NPTII activity from the full length promoter in parsley protoplasts (compare Figures 2b and 3). Moreover, quantitative levels (fold induction) of light activated CHS mRNA expression in *A. majus* seedlings and the full promoter construct in heterologous protoplasts are of the same order of magnitude. The homologous parsley CHS is also light responsive as part of a chimeric gene fusion in parsley protoplasts in a manner reflecting the endogenous gene (Dangl *et al.*, 1987; Schulze-Lefert *et al.*, 1988). Finally, we find low level light independent CHS expression in both *A. majus* seedlings and with the full promoter (construct 714) in heterologous protoplasts (see Figures 1–3). This is consistent with the recently described developmental regulation of CHS in parsley seedlings and etiolated leaves on adult plants re-exposed to light (Jahnen and Hahlbrock, 1988; Schmelzer *et al.*, 1988; see also Dangl *et al.*, 1988). Together these

points validate the use of heterologous parsley protoplasts for promoter analysis of the *A. majus* *chs* gene.

We stress that this type of transient assay system relies on appropriately responding protoplasts and on a gene whose control in the homologous species is reasonably well characterized. Though these criteria have been met in the many analyses of other light regulated genes in transgenic plants (for reviews see Kuhlemeier *et al.*, 1987a,b; Schell, 1987), ours is the first example of inducible transient expression of a light regulated gene in heterologous protoplasts. To date, this work, a parallel analysis of the homologous parsley CHS promoter (Schulze-Lefert *et al.*, 1988) and an account of anaerobic induction (Howard *et al.*, 1987) are the only cases of inducible transient gene expression in protoplasts. In the latter case (Howard *et al.*, 1987), a maize *Adh-2* promoter construct was regulated by anaerobiosis in homologous protoplasts derived from suspension cultured cells. Whether or not the rapid, position effect independent promoter analysis offered through this method can be generally applied to other induction mechanisms (e.g. pathogen, salt or heat stress) remains to be determined, although its utility in analysis of enhancers has been shown (Ellis *et al.*, 1987).

Three distinct *cis*-elements contribute to CHS promoter light response

Our assay reveals three types of regulatory elements. One of these is directly responsive to UV light while the other two modulate the quantity of expression.

The smallest fragment which maintains low level light responsiveness is immediately proximal to the TATA box (-39 to -197 ; construct $\Delta 5$, see Figure 5). This fragment functions when inverted (714R and $\Delta 7R$, Figure 7a) and renders a heterologous minimal promoter UV-light responsive (Figure 7b). Only a construct containing three tandem copies of this fragment was tested in the latter case and it remains to be determined whether or not a single copy is sufficient to mediate UV-light activated expression. Removal of this fragment leads in all cases to complete abrogation of UV-light activation.

A TATA proximal location for the CHS UV-light responsive element is consistent with other light regulatory elements from genes encoding SSU and CAB (Kuhlemeier *et al.*, 1987a,b; Nagy *et al.*, 1987; Castresana *et al.*, 1988; Simpson *et al.*, 1986). Moreover, Schulze-Lefert *et al.* (1988) recently demonstrated a functional requirement for two UV-light inducible *in vivo* DNA footprints located between -130 and -170 on the parsley CHS promoter. A degenerate copy of one of these elements is present in the *A. majus* CHS promoter at -70 and may be important. Sommer *et al.* (1988) elegantly defined a quantitative role for a direct repeat of the hexamer TACCAT in *A. majus* plants. These elements are located at -55 and -43 and their deletion through transposon excision drastically affects steady-state mRNA levels. Although presence of these sequences may play a role in maintaining high level expression under light conditions, their involvement in the initial response to UV light is unknown.

Sequences between -197 and -357 enhance specifically UV-light activated expression only when the minimal responsive fragment is present (compare $\Delta 2$ and $\Delta 5$, Figure 5). When present alone, these sequences support only low level expression. Refined analysis of this region is required

to define the sequences which work in concert with the minimal UV-light element. Since various wavelengths of light modulate CHS transcription rate (Ohl *et al.*, 1988) it could be the case that either the light dependent enhancer or the TATA proximal fragment, or both, are responsive to visible wavelengths.

Finally, our data clearly indicate the presence of a general, light independent enhancer between positions -564 and -661. This sequence affects levels of transcription on both UV-light treated and control protoplasts, though it alone is not light responsive. As well, it can be moved ~370 bp and retains its activity (714 compared to $\Delta 8$, Figure 5). Protein derived from tobacco seedling nuclear extracts binds to this fragment (Kaulen, 1986; D.Staiger and J.Schell, unpublished).

The complexity of control mechanisms governing UV-light regulated expression from the CHS promoter follows the trend set through analysis of other light responsive genes. The use of a UV-light responsive transient assay system allowed us to rapidly define fragments determining both qualitative and quantitative features of CHS light activation. Extension of these results will provide a more refined understanding of the activation of the key gene involved in flavonoid synthesis. Moreover, analysis of light qualities effecting CHS expression may help to dissect the potentially overlapping regulatory networks shared by the *chs* gene and the more thoroughly analysed light responsive genes encoding the SSU of ribulose biphosphate decarboxylase and chlorophyll A/B binding protein.

Materials and methods

Recombinant DNA techniques

Cloning steps were performed according to the protocols of Maniatis *et al.* (1982). The fragments were cut out with indicated restriction enzymes, gel purified and ligated into polylinker derivatives of pUC19.

Cloning vectors were derivatives of pUC18 and pUC19 (D.Wing; R.Töpfer, Köln). *Escherichia coli* strain K12 MC1061 (Casadaban *et al.*, 1980) was used for cloning and DNA preparation. For chimeric 714 and $\Delta 1-\Delta 4$ constructs see Kaulen *et al.* (1986). New constructions were generated as follows.

714 pUC contains the complete chimeric 714 construct as an *EcoRI*-*SalI* fragment in the polylinker of pUC19. This construct was digested with *EcoRI* and *KpnI* and the vector fragment isolated as a receptor for new promoter fragments. To construct $\Delta 5$, the *KpnI*-*KpnI* fragment of position -197 to -39 was subcloned into a pUC19 derivative and subsequently excised by complete *EcoRI* and partial *KpnI* digestion and ligated into the receptor vector. The *HincII*-*KpnI* fragment of position -357 to -197 was similarly cloned. $\Delta 3$ was linearized with *KpnI* and ligated to an isolated *KpnI* fragment isolated from the full promoter, resulting in $\Delta 7$ and $\Delta 7R$, an internal inversion. The inversion of the *KpnI* fragment in 714 was obtained in a similar way.

The *SstI* site of the pUC linker in $\Delta 5$ was opened, blunt ends generated and the vector recut by *EcoRI*. Into this vector, the fragment of position -661 and -564 (provided by Dr D.Wing) was ligated as a 5' *EcoRI* and 3' *SalI* filled-in fragment to give $\Delta 8$.

All chimeric genes were subcloned as *EcoRI*-*BamHI* fragments into pSL2, a pUC18 derivative described below.

FHEI714Nos contains the *chs*-5' upstream promoter region from -920 to -39 in front of the shortened nopaline synthase (NOS) promoter. To construct this plasmid the 780 bp *MsrII*-*SphI* fragment of pLGVneo1103 which contains the *nos* promoter from -80 to the transcription start site, including a CAAT box and a TATA box, and part of the *npII* gene was subcloned in pUC19. pUC19 was linearized with *BamHI*, filled-in and recut with *SphI*. The subcloned *DraI*-*SmaI* CHS promoter fragment (D.Wing) was ligated into the *SmaI* side of the pUC polylinker in front of the minimal *nos* promoter. To complete the *npII* gene, the promoter fusion was isolated after digestion with *EcoRI* and *SphI* and subcloned in FHEI. Subsequently pFHEI714CR (Kaulen *et al.*, 1986) was digested with *EcoRI* and *SphI* and the vector was isolated and ligated with the *EcoRI*-*SphI* 714Nos promoter fragment.

To regenerate pFHEIMiNos the *chs* promoter fragment was cut out of pFHEI714Nos with *KpnI*. pFHEI 5Nos was constructed by ligating the *KpnI* fragment from -39 to -197 into the *KpnI* site of pFHEIMiNos. Unfortunately, the subclones carried three tandem repeats of the insert.

To construct the high copy vector pSL2, a pUC18 derivative of pRT99neo (Töpfer *et al.*, 1988) was used. It carries at position 1554 a 35S cauliflower mosaic virus (CaMV) promoter and the CaMV polyadenylation signal and the structural glucuronidase (GUS) gene of pTAK1 (Jefferson *et al.*, 1987) was inserted. The complete 714 chimeric NPTII construction went as a 2.84 kb *EcoRI*-*SalI* fragment into the polylinker to form pSL2.714 (7.8 kb). From this construct the promoter region was excised by unique *EcoRI*-*BamHI* digestion and all promoter mutants went *EcoRI*-*BamHI* into pSL2 except $\Delta 4$ and the *nos* constructs. No influence of the CaMV promoter on the *chs*-*npII* gene was observed.

Plasmid DNA for transformation was prepared by the cleared lysate method (see Maniatis *et al.*, 1982) and was twice banded in a CsCl gradient, butanol extracted, dialysed and ethanol precipitated. DNA was routinely adjusted to 1 mg/ml in TE.

Plant material and transient expression

Parsley suspension cultures (*Petroselinum crispum*) were grown in the dark in modified B5 media and subcultured as described (Hahlbrock, 1976). The cell culture used for protoplast preparation was 2 or 5 days old without aberrant differences. Protoplasts were prepared by incubation of 400 mg fresh weight in 12 ml 0.25% cellulase RS and 0.05% mazerzyme in 0.24 M CaCl_2 for 14 h at 26°C and purified as described in Dangel *et al.* (1987). Finally protoplasts were adjusted to $5 \times 10^6/\text{ml}$ in 0.24 M CaCl_2 .

Transformation of parsley protoplasts (ppp)

This was done via the $\text{Ca}(\text{NO}_3)_2$ PEG method (Krens *et al.*, 1982; Hain *et al.*, 1985). Drops of 400 μl (2×10^6 ppp) were mixed with 40 μl of DNA solution (20 $\mu\text{g}/10^6$ ppp) and 400 μl of PEG solution [0.1 M Hepes, 25% PEG 6000 (BDH), 0.45 M mannitol, 0.1 M $\text{Ca}(\text{NO}_3)_2$] were added. After 10 min incubation, 10 ml washing solution [0.275 M $\text{Ca}(\text{NO}_3)_2$] was added stepwise and carefully mixed with the protoplasts. The ppp were pelleted 5 min later by 3' centrifugation at 60 g. The pellet was resuspended in 6 ml B5 media (14% sucrose, 680–700 mOs, 1 mg 2,4 D/1) and plated in two 6-cm Petri dishes. One dish was sealed in aluminium foil as a dark control. Both samples were placed into the light induction chamber.

UV irradiation

This was performed on a white plastic surface for the indicated times with a continuous white light including wavelengths 300–400 nm, as described in Dangel *et al.* (1987). After induction, protoplasts were harvested by diluting the culture media in 3–4 volumes 0.24 M CaCl_2 , centrifuged for 5 min at 100 g, aspirated and the pellet was resuspended, transferred to an Eppendorf tube, and pelleted for 30 s at 8000 g. The fresh weight was determined (35–45 mg/ 10^6 protoplasts after 15 h) and samples were frozen in N_2 and stored at -70°C.

Testing various transformation conditions, we found that 10–20 μg of DNA was optimal. Higher amounts did not result in better inducibility; lower amounts of DNA gave lower overall expression levels. Preliminary transient expression experiments with pUC714, pFHEI714 and pSL2714 resulted in comparable expression data indicating that the 35S CaMV promoter of the *gus* gene present in pSL2 does not obviously effect the CHS-NPTII induction.

Enzyme assays

The *in situ* NPTII gel assay for plants (Reiss *et al.*, 1984) was performed with slight modifications. The native acrylamide slab gel was run for 4–5 h at 80 mA. The active fraction co-migrates with Xylene-cyanol dye and was cut as a 6-cm gel strip. GUS activity was determined by the protocol of Jefferson *et al.* (1987).

A. majus

A. majus (Snapdragon) was the commercial variety Tip-top Red flowering. Seeds were either grown in soil and covered with a 1.5-cm plexiglass shield or they were surface sterilized, placed onto a wet 3MM chromatography paper in plastic dishes and wrapped with aluminium foil. Seedlings were UV irradiated. Adult plants were roughly 2 months old, 25-cm high and had not started to flower.

Purification and analysis

RNA purification (Logemann *et al.*, 1986) and Northern analysis followed established protocols. The probe, the cDNA of *A. majus chs* gene (1.1 kb) was a gift of H.Sommer, Köln, and labelled with [^{14}C]dCTP by multiprime kit (Amersham).

Variability of inducible transient expression

We and others (Schulze-Lefert *et al.*, 1988) observed variations in the overall transcriptional fitness of parsley protoplasts between different preparations. Calculation of an 'induction factor' relies on measurements of very low, and therefore statistically intrinsically inaccurate, dark control values. It is just this background level which can vary by 2- to 3-fold from week to week. To overcome this problem, five NPTII tests, from a total of 36, were quantitated by counting, and comparisons between constructs are only made **within** an experiment. A second constitutive marker on the same plasmid, the 35S promoter of CaMV driving the GUS reporter gene, was also used to assess gene transfer efficiencies. It is difficult, however, to 'correct' NPTII activity data using GUS activity as a normalizer, since UV-light induction of constructs appeared to always influence expression from the CaMV 35S promoter linked on the same plasmid. It is critical to note that in our transformation protocol both dark and UV-light treated samples are derived from the same transformation event. Thus, GUS activity from the dark aliquot was used to show that all transformation efficiencies within an experiment only varied by 10%.

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