The EMBO Journal vol.5 no.1 pp.1–8, 1986

Light-induced expression of the chimeric chalcone synthase-NPTII gene in tobacco cells

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Communicated by J.Schell

A chimeric gene was constructed containing the light-inducible chalcone synthase (*chs*) promoter from *Antirrhinum majus*, the neomycin phosphotransferase structural sequence from Tn5 as a reporter gene (*NPTII*) and the termination region from *chs* gene 1 from *Petroselinum hortense*. This gene was introduced into tobacco plants with the help of Ti plasmid-derived plant vectors and *NPTII* expression was measured. Analysis of the *chs* promoter sequence indicated the position of several possible regulatory regions. These were deleted to test their influence on the expression of the *chs-NPTII* gene. The different chimeric genes were all shown to be active after transfer to tobacco with the exception of one, in which the entire 5′ upstream sequence from −1200 to −39 was deleted. The transcription of a chimeric gene with a 1.2-kbp 5′ upstream promoter sequence was shown to be light inducible in tobacco plants. The analysis of various deletions of this 5′ upstream sequence indicates that a number of sequence motifs have a quantitative effect on gene expression. One of these sequence motifs (−564 to −661) contains a direct repeat of 47 bp and the sequence GTGGTTAG which corresponds to the consensus core sequences observed in animal gene enhancer sequences. Deletion of a fragment containing this direct repeat resulted in a reduction of *NPTII* expression by a factor of 5.

Key words: chalcone synthase-NPTII gene/light induction/promoter analysis/Ti plasmid

Introduction

Light is one of the most important effectors for differentiation in plants (Mohr and Schopfer, 1977, 1983). It regulates the switch in plant development from scotomorphogenesis (light is lacking) to photomorphogenesis (light affluence). Three different sensory pigments are known to control differentiation processes induced by light in higher plants. The first and best characterized photoreceptor is phytochrome, operating predominantly in the red/far red spectral range (Siegelman and Hendricks, 1965; Siegelman *et al.*, 1966; Rüdiger and Corell, 1969). The second photoreceptor system involved in photomorphogenesis is the blue light photoreceptor (cryptochrome), which seems to be a flavone, probably a flavoprotein (Gressel, 1979). The third sensor pigment known to occur in higher plants is the u.v.-B photoreceptor whose action spectrum shows a maximum at 290 nm (Wellmann, 1971, 1974; Yatsuhashi *et al.*, 1982).

One of the most intensively studied regulatory effects of light on specific metabolic pathways is the induction of flavonoid biosynthesis (Kreuzaler *et al.*, 1983; Schröder *et al.*, 1979; Wellmann and Schopfer, 1975). Flavonoids are abundant in all higher plants and serve important functions such as flower pigments, u.v.-protective compounds and antimicrobial substances (phytoalexins) (Grisebach and Ebel, 1978; Albersheim and Valent, 1978). The enzyme chalcone synthase (*chs*) catalyses the stepwise condensation of acetate from malonyl-CoA with the starter molecule 4-cumaroyl-CoA to give the naringenin chalcone (Kreuzaler and Hahlbrock, 1975a, 1975b; Kreuzaler *et al.*, 1978, 1979; Heller and Hahlbrock, 1980) the central intermediate in the biosynthesis of flavonoids. Induction of expression of the flavonoid genes can vary in different cells. In some plants biosynthesis is regulated primarily by phytochrome as in *Antirrhinum majus* during a defined and short period of seedling development (E. Wellmann, personal communication), whereas in others, u.v.-A or u.v.-B is also required in the induction process (Downs and Siegelman, 1963; Drumm and Mohr, 1974). A cDNA clone from the key enzyme of the flavonoid glucoside pathway, the chalcone synthase (*cDNAchs*) from *Petroselinum hortense* (Kreuzaler *et al.*, 1983) was used to identify a genomic clone from *A. majus* (Wienand *et al.*, 1982). We have used the 5′-upstream region of the chalcone synthase gene from *A. majus* (Sommer and Saedler, 1986) and the coding sequence of neomycin phosphotransferase (NPTII) from Tn5 (Beck *et al.*, 1982) to construct chimeric genes which were integrated into the genome of tobacco cells. Expression of these genes in the non-homologous host was tested. Here we present data showing that the *chs-NPTII* gene is expressed in tobacco cells and that u.v.-B light induces gene expression. The type of expression of the *chs-NPTII* gene is similar to that observed for chalcone synthase in cell suspension cultures from *P. hortense*. Some areas within the promoter which are of potential importance for the expression of the *chs-NPTII* gene have been identified.

Results

Construction of the plasmid pFHEI710CR containing the chimeric chalcone synthase NPTII gene (chs-NPTII)

The chalcone synthase cDNA from *P. hortense* (Kreuzaler *et al.*, 1983; Reimold *et al.*, 1983) was used as a probe to isolate the chalcone synthase genomic clone from *A.majus*. This genomic clone, which was a gift from H. Sommer, contains a 3.9-kbp region upstream from the 5′ end of the chalcone synthase structural gene. This DNA fragment was used as a putative promoter for the construction of a chimeric gene (Figure 1). We assumed that this region contains the sequences essential for regulation of transcription. The DNA fragment was ligated to the 5′ end of the NPTII structural gene (Beck *et al.*, 1982). As shown in Figure 2, the *chs* promoter fragment contains the 5′-untranslated region of the *chs* gene and an ATG, which should be used as the initiation codon for translation. In order to add a *Bam*HI restriction site to the 3′ end of the promoter fragment, an original *Ava*II site in the *chs* structural gene close to the ATG was treated with S1 nuclease to generate blunt ends. The area was resequenced to identify the exact 3′ end. The S1 nuclease had removed 13 nucleotides from the original *Ava*II site. To achieve a correct reading frame, we had to use the NPTII coding region of plasmid pKm 109/9 (Reiss *et al.*, 1984a; for further details see Figure 1). As shown in Figure 2, the NPTII protein from the *chs-NPTII*

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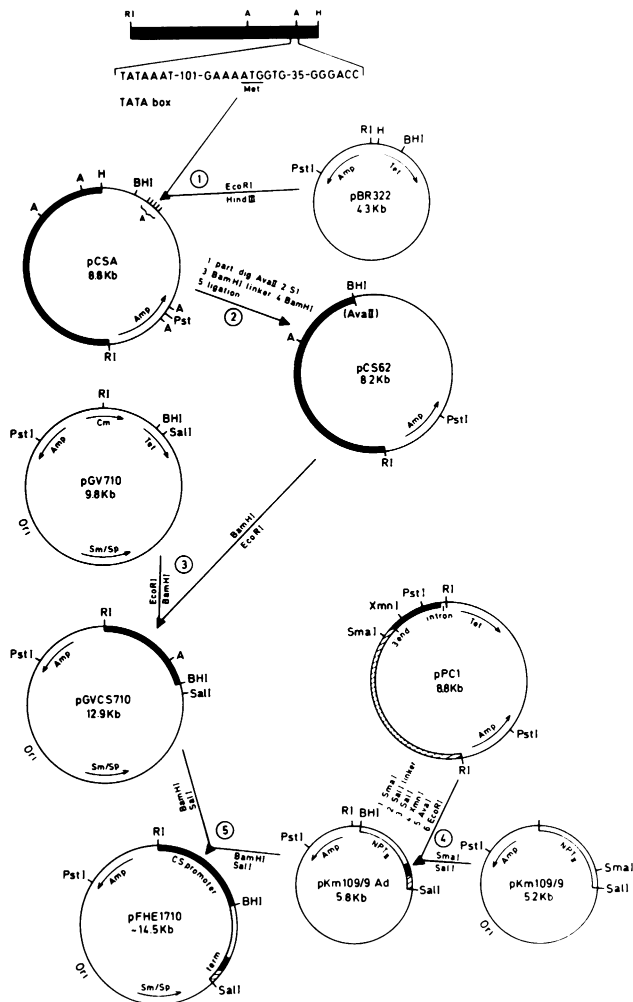

Fig. 1. Construction of the plasmid pFHEI710CR containing the *chs-NPTII* gene. 1. The 4.5-kbp fragment (black bar; *Eco*RI-*Hind*III) containing the 5′ upstream sequences of the chalcone synthase gene from *A. majus* was integrated into pBR322 (pCSA; 8.8 kbp). 2. The *Ava*II restriction site near the *Hind*III site was changed to a *Bam*HI site. pCSA was partially digested with *Ava*II in the presence of ethidium bromide (20 μg/ml) and then treated with S1 nuclease under mild conditions (Law *et al.*, 1980). A *Bam*HI linker was ligated to the blunt ends and the plasmid was self-ligated (pCS 62; 8.2 kbp). 3. The *Eco*RI/*Bam*HI fragment from pCS62 containing the 5′ upstream sequences was integrated into pGV710 to give the resulting plasmid pGVCS710 (12.9 kbp). 4. The plasmid pPCl (8.8 kbp) containing the 3′ end of the chalcone synthase gene I (4.5 kbp) from *P. hortense* was first cut with *Sma*I. The *Sma*I restriction site is located ~400 bp downstream from the 3′ end of the *chs* structural gene. A *Sal*I linker was added to the blunt ends and the DNA was cut with *Xmn*I. In addition, the DNA was digested with *Ava*I and *Eco*RI to cleave DNA fragments which could also have been integrated into pKm 109/9 (5.2 kbp). pKm 109/9 containing the NPTII gene on a *Bam*HI-*Sal*I fragment was cut with *Sma*I and *Sal*I and the DNA of pPCl and pKm 109/9 was ligated. The resulting plasmid pKm 109/9 Ad (5.8 kbp) was confirmed by restriction mapping and by Southern hybridization using the *chs* fragment from parsley as a probe. 5. pKm 109/9 Ad was cut with *Bam*HI and *Sal*I and the fragment containing the NPTII gene was integrated into the *Bam*HI-*Sal*I site of plasmid pGVCS710. The resulting plasmid is pFHEI710CR (14.5 kbp) containing the chimeric gene. RI, *Eco*RI; A, *Ava*II; H, *Hind*III; BHI, *Bam*HI; Amp, ampicillin; Tet, tetracycline; Ori, origin of replication; Cm, chloramphenicol; Sm, streptinomycin; Sp, spectinomycin; S1, S1 nuclease; intron, the cloned *chs* DNA (4.5 kbp) contains a 60-bp intervening sequence; 3′ end, 3′ end of the *chs* structural gene; term, termination region of *chs* gene I from parsley.

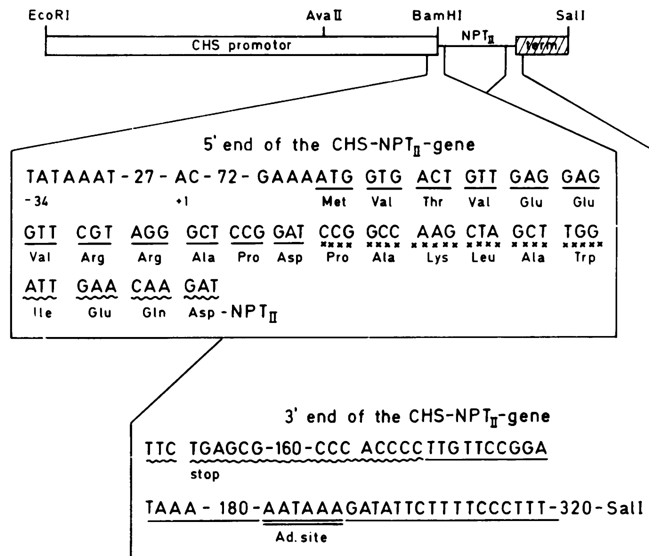

Fig. 2. Structure of the *chs-NPTII* gene. At the 5′ end the *chs-NPTII* gene contains the untranslated leader of the *chs* structural gene, its ATG and 11 amino acids of the *chs* coding region (underlined), which are in-frame with the NPTII sequence (wavy line). The linker region between both is marked with stars. At the 3′ end of the *chs-NPTII* gene the sequence marked with a wavy line belongs to the NPTII gene. The underlined sequence is derived from the 3′ end of the *chs* gene I from *P. hortense* fused to the NPTII gene as a termination region. Ad site = polyadenylation site.

gene will be 18 amino acids longer than a protein coded by the sequence from the plasmid pKm 109/9.

The unmodified NPTII protein confers resistance to plants against the antibiotic kanamycin and modification of the amino-terminal end does not necessarily change the capacity of this enzyme to transfer a phosphate group from ATP to kanamycin (Reiss *et al.*, 1984a; Herrera-Estrella *et al.*, 1984; Fraley *et al.*, 1983; Bevan *et al.*, 1983; Schreier *et al.*, 1985). We therefore expected the *chs-NPTII* fusion protein to show a similar activity. This was established by injecting the construction into the nucleus of the algae *Acetabularia* (Neuhaus *et al.*, 1984) where the gene was expressed, producing cells resistant to the antibiotic G418 (Gibco) (G.Neuhaus *et al.*, in preparation).

It has been shown that the 3′-downstream sequences can be of importance for optimal expression of a gene (Georgiev and Birnstiel, 1985). We therefore decided to ligate a 600-bp fragment from the 3′ end of the chalcone synthase gene 1 from *P. hortense* in the correct orientation to the 3′ end of the *chs-NPTII* gene (Figures 1 and 2). This fragment contains 217 bp from the 3′ end of the coding region of the chalcone synthase gene and ~450 bp downstream from the end point of transcription. The chimeric gene construction was integrated into the plasmid pGV710 (Deblaere *et al.*, 1985). The resulting plasmid pFHEI710CR contains the spectinomycin (Sp) gene which is needed to identify colonies of *Agrobacterium tumefaciens* harbouring the chimeric gene.

Construction of 5′ upstream deletion mutants of the chalcone synthase NPTII gene

The *chs-NPTII* gene containing the 3.9 kbp long 5′ upstream region was chosen to represent a wild-type situation and from this a series of deletion mutants were constructed (Figure 3). Analysis of DNA sequences of the *chs* 5′ upstream region had

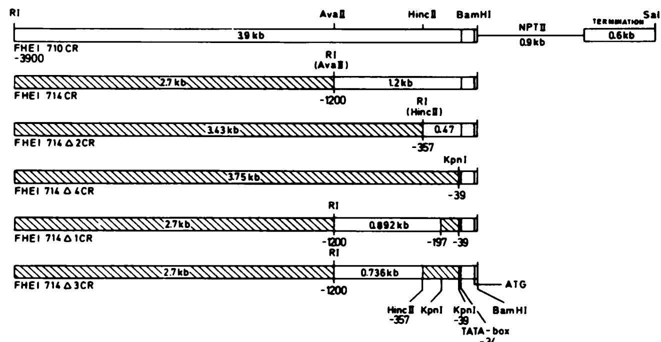

Fig. 3. Construction of the *chs-NPTII* deletion mutants. The 3.9-kbp *Eco*RI/*Bam*HI fragment containing the *chs* promoter from *A. majus* in pFHEI710CR represents a wild-type situation. To generate 5′ deletion mutants, the promoter was shortened to the end points −1200, −357 and −39. The TATA box is located at −34. To create the 1.2-kbp promoter fragment, an *Ava*II site at −1200 was treated with S1 nuclease and an *Eco*RI linker was added to the blunt ends, to give plasmid pFHEI714CR. To generate the deletion mutant pFHEI714Δ2CR, the 1.2-kbp promoter was subcloned into the polylinker of pUC9 as an *Eco*RI-*Bam*HI subfragment, followed by digestion with *Hinc*II, addition of an *Eco*RI linker to the blunt ends, recutting with *Eco*RI and *Bam*HI and inserting the truncated promoter into the plasmid pFHEI714CR. The deletion mutant with its end point at −39 was created by cutting the plasmid pFHEI714CR with *Eco*RI, filling in the staggered ends with DNA polymerase I Klenow fragment and adding a *Kpn*I linker. This could then be recut with *Kpn*I and recircularized with T4 ligase resulting in the mutant pFHEI714Δ4CR. The internal deletion pFHEI714Δ1CR was generated by cutting the plasmid pFHEI714CR with *Kpn*I, removing the 158-bp fragment between the two *Kpn*I sites at −39 and −197 with an isopropanol precipitation and religating the plasmid with T4 ligase. The 318-bp internal deletion was created using the 1.2-kbp promoter subcloned in pUC9. The *Hinc*II site in the polylinker was eliminated by cutting with *Sal*I followed by a fill-in reaction using DNA polymerase I Klenow fragment. The resulting plasmid pUC9714ΔSa1CR (not shown) was used for further manipulation to create the deletion. This was done by linearization with *Hinc*II, adding a *Kpn*I linker to the blunt ends, cutting with *Kpn*I and religating to give the final deletion mutant pFHEI714Δ3CR. All the hatched areas in Figure 3 are the deleted ones.

shown several remarkable sequences located in three regions upstream from the TATA box (Sommer and Saedler, 1986). The first is located close to the TATA box between positions −38 and −70, the second between positions −270 and −340 and the third upstream of position −550. To test, whether these sequences might have some influence on expression of the *chs-NPTII* gene, we deleted them to construct mutated promoters. The DNA fragment containing the 3.9-kbp *chs* promoter region was shortened to 1.2 kbp (pFHEI714CR) and to 0.357 kbp (pFHEI714Δ2CR). This was done by changing the *Ava*II site at −1200 and the *Hinc*II site at −357 into an *Eco*RI site. Two *Kpn*I sites are located in the *chs* promoter close to the TATA box, which is located at position −34 [*Kpn*I(1) −39; *Kpn*I(2) −197]. These *Kpn*I sites were used to create an internal deletion. A 158-bp internal DNA fragment was deleted from the promoter of the *chs-NPTII* 5′ deletion mutation pFHEI714CR to give the resulting plasmid pFHEI714Δ1CR. The 3′ end of the deleted fragment is 5 bp upstream from the TATA box. Another internal deletion (pFHEI714Δ3CR) was constructed by deleting a 318-bp fragment located between the positions −39 and −357 (*Kpn*I-*Hinc*II). To test whether the 5′ upstream sequences of the *chs-NPTII* gene are essential for gene expression, we constructed a truncated promoter in which the whole region between positions −39 and −1200 is deleted (pFHEI714Δ4CR). The TATA box was kept intact in all constructions.

Introduction of the chs-NPTII gene into tobacco cells

To introduce the chimeric genes into the genome of plants, the plasmids containing the different constructions were inserted into the pBR322 DNA of pGV3850 or pGV3851. These Ti plasmids are derivatives of pTIC58. In these vectors most of the T-DNA is substituted by pBR322, which is transferred and integrated into nuclear DNA (Zambryski *et al.*, 1983).

The different constructions were introduced into the Ti plasmids pGV3850 and pGV3851 by homologous recombination after mobilization from *Escherichia coli* to *A. tumefaciens* with the help of the plasmids R64drd11 and GJ28 (Van Haute *et al.*, 1983; Koncz *et al.*, 1984). Since all chimeric genes were integrated into the plasmid pGV710, co-integrates with the Ti plasmid vectors were selected on plates containing spectinomycin and streptomycin. The structures of the chimeric genes were verified by Southern blot hybridization, using the NPTII gene and the chalcone synthase promoter region as probes. It was shown that the genes were integrated into the T-DNA without any detectable rearrangement (data not shown).

Transformed teratoma tissue obtained by inoculation with *Agrobacteria* containing the chimeric genes in the Ti plasmid pGV3851 were screened for the presence of nopaline synthase activity as a co-transferred marker (Otten, 1982). Several independent teratoma lines were obtained for every construction. Tobacco cells transformed with the chimeric genes integrated in the Ti plasmid pGV3850 (pGV3850::FHEI710CR, FHEI714CR, FHEI714Δ1CR, FHEI714Δ2CR, FHEI714Δ3CR, FHEI714Δ4CR)) were regenerated to intact plants (see Materials and methods). Transformed plants were identified by testing for the co-transferred nopaline synthase. Seeds of intact transformed plants were obtained by selfing. One plant (plant C, containing the 1.2-kbp promoter construction) was chosen for further experiments. The F1 progenies of plant C expressed the nopaline synthase gene and segregated in a 3:1 ratio (transformed versus untransformed). This result makes it very likely that an active gene is integrated into the plant genome as a single locus and that this gene is inherited as a dominant Mendelian trait.

Expression of the chimeric genes in tobacco cells

Expression of the *chs-NPTII* gene (FHEI714CR) was measured in teratoma tissue, intact plants and protoplasts (transient expression). By the neomycin phosphotransferase assay we established that the NPTII protein was present in crude extracts isolated from teratoma cells transformed with FHEI714CR. No neomycin phosphotransferase activity could be found in untransformed tobacco tissue. The method used to detect the NPTII activity was similar to a published procedure (Reiss *et al.*, 1984b; Schreier *et al.*, 1985). The results of a typical NPTII test showed that the translation product of the *chs-NPTII* gene is, as expected, 18 amino acids longer than the coding sequence from plasmid pKm 109/9 (Figures 2 and 4). The same result was obtained with a protein extract from intact plants, transformed with either the chimeric genes FHEI710CR or FHEI714CR. Experiments were done to test whether or not the chimeric genes were expressed in protoplasts (transient expression, see Materials and methods). The plasmid pGV3850::1103neo containing the neomycin phosphotransferase gene under the control of the nopaline synthase promoter, was used as a control to test the transcription capacity of the protoplasts. The results showed that the *nos-NPTII* gene was expressed as expected, whereas no NPTII activity was detected in protoplasts transformed with the *chs-NPTII* gene (data not shown).

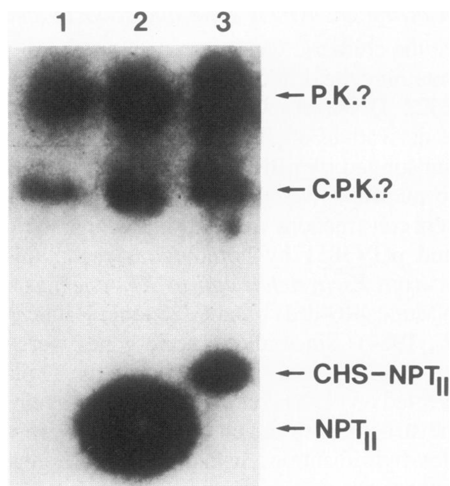

Fig. 4. Demonstration of the *chs-NPTII* fusion protein in transformed teratoma tissue using the neomycin phosphotransferase assay. **Lane 1,** untransformed *N. tabacum* Wisconsin 38. **Lane 2,** NPTII activity in crude extracts from transformed callus material derived after co-cultivation with the *Agrobacterium* strain C58Cl pGV3850::pAK1003 (kindly provided by J. Velten). This construction contains the *NPTII* gene under the control of the *1'* TR promoter (Velten *et al.*, 1984). The translation product represents an unmodified NPTII protein. **Lane 3,** NPTII activity in crude extracts from teratoma material transformed with the *Agrobacterium* strain C58Cl pGV3851::pFHEI714CR, harbouring the 1.2-kbp *chs* promoter fragment. The *chs-NPTII* fusion protein shows a shift of the enzymatic activity to a position of reduced electrophoretic mobility. The P.K. ? band is presumed to be due to a cytoplasmic self-phosphorylating protein and the C.P.K. ? is presumed to be due to a chloroplast self-phosphorylating protein.

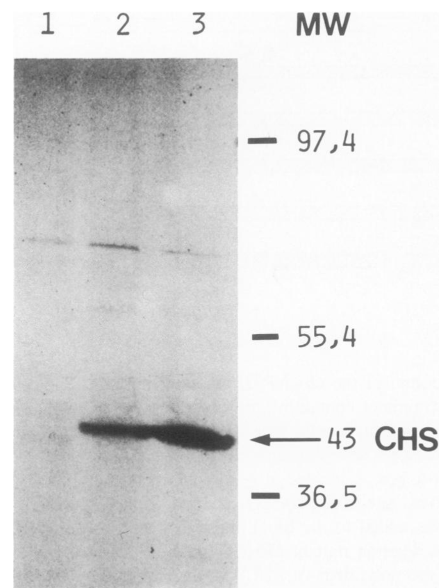

Fig. 5. Detection of the *chs* protein by immunoblotting in plant extracts of *N. tabacum* after induction of the plants with sunlight. Crude extracts of 30 μg total protein were separated by size on a polyacrylamide slab gel according to Laemmli (1970) and electrophoretically blotted to nitrocellulose. The filter was treated with affinity purified anti-*chs* at 7.5 μg/ml. Staining was done using peroxidase-labelled sheep anti-rabbit antibodies. **Lane 1,** crude extracts from 6-week old *N. tabacum* W38 plants not induced with sunlight. **Lane 2,** crude extracts from 6-week old *N. tabacum* W38 plants induced with sunlight for 24 h. **Lane 3,** crude extracts from 6-week old *N. tabacum* W38 plants germinated in sterile culture on LS medium and transferred after germination to soil in the greenhouse induced by sunlight for 24 h.

Expression of the endogenous chalcone synthase in tobacco cells

Induction of expression of the endogenous *chs* gene can be achieved in tobacco plants by sunlight or u.v.-B at 310 nm. This was shown by immunoblotting using affinity-purified monospecific antibodies as a probe (Kreuzaler *et al.*, 1979). Young plants (4–6 weeks after germination) grown in the greenhouse were protected against u.v.-B light with a plastic shield. When these plants were subjected to sunlight or illuminated with u.v.-B for 24 h, the presence of the *chs* protein could be demonstrated (Figure 5). Older plants from the greenhouse do not show any *chs* protein in the leaves. However, even these plants expressed the *chs* gene after induction with sunlight for ~24 h. The only organ in which the chalcone synthase protein could be detected in greenhouse material without u.v. induction were the petals. Expression of the *chs* gene in flowers might be hormone induced (E. Wellmann, personal communication).

5' Upstream regions which influence expression of the chs-NPTII gene

To analyse which sequences of the *A. majus* promoter modulate expression of the *chs-NPTII* gene, the NPTII activity of all constructions was measured under identical conditions. The experiments were performed with teratoma cells grown in a cycle of 16 h white light and 8 h darkness. To minimize any possible position effects, several independent teratoma lines were pooled, the protein fraction was isolated and the expression of the different *chs-NPTII* genes was measured using the neomycin phosphotransferase assay (Reiss *et al.*, 1984b; Schreier *et al.*, 1985). An example is shown in Figure 6.

The results of many assays were averaged and are presented as the following percentages. The chimeric gene containing the 1.2-kbp promoter (FHEI714CR) was expressed at the highest rate (100%). Expression of the construction FHEI710CR, under the control of the 3.9-kbp promoter, was ~80%. An important drop in gene expression can be seen, when the promoter region was shortened to 357 bp (FHEI714Δ2CR). In this case the *chs-NPTII* gene was expressed at 20%, compared with the 1.2-kbp promoter of gene FHEI714CR.

In the *chs-NPTII* gene FHEI714Δ1CR, a fragment of 158 bp was deleted from the promoter of pFHEI714CR (1.2 kbp). The 3' end of this fragment is located only 5 bp upstream from the TATA box. Elimination of this fragment reduced the level of expression to 40%.

This reduction is even more pronounced when a fragment of 318 bp, located 5 bp upstream from the TATA box, was deleted (FHEI714Δ3CR). Expression of this construction was only ~25%. No enzymatic activity could be demonstrated in cells transformed with the chimeric gene FHEI714Δ4CR. The promoter of this construction is only 39 bp long, but it contains an intact TATA box and, therefore, transcription could start at the correct point of initiation. The observation that expression of the chimeric gene FHEI714Δ4CR cannot be detected in teratoma tissue is supported by experiments in which we tested growth of the calli on various concentrations of kanamycin. Calli containing the chimeric genes FHEI710CR, FHEI714CR, FHEI714Δ1CR, FHEI714Δ2CR and FHEI714Δ3CR could grow on kanamycin concentrations of up to 250 μg/ml, a concentration at which untransformed cells died. However, teratoma cells transformed with FHEI714Δ4CR did not survive on agar plates containing 75 μg/ml of the antibiotic.

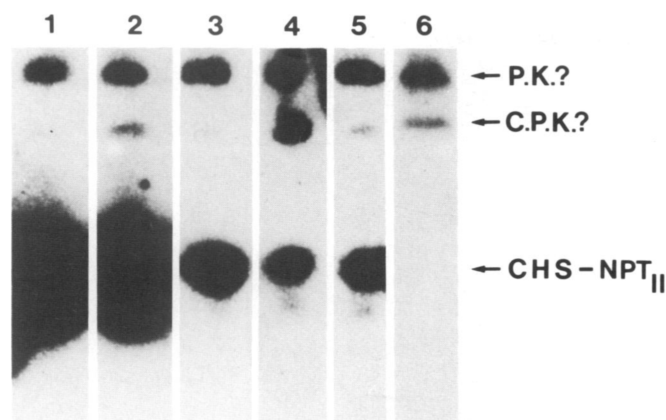

Fig. 6. Comparison of the NPTII activity in the various *chs-NPTII* constructions. For each construction several independent teratoma lines were pooled, the protein fraction was isolated and 30 μg of total protein was loaded on a non-denaturing polyacrylamide gel, so that the lanes on the gel are comparable. The NPTII assay was performed as described in Materials and methods. **Lane 1,** NPTII activity in crude extracts from teratoma material harbouring the chimeric gene plasmid pFHEI710CR (3.9-kbp promoter fragment). **Lane 2,** crude extracts from teratoma material harbouring the plasmid pFHEI714CR (1.2-kbp promoter fragment). **Lane 3,** crude extracts from teratoma material harbouring the plasmid pFHEI714Δ2CR (357-bp promoter fragment). **Lane 4,** crude extracts from teratoma material harbouring the plasmid pFHEI714Δ1CR (158-bp internal deletion 5 bp in front of the TATA box). **Lane 5,** crude extracts from teratoma material harbouring the plasmid pFHEIΔ3CR (318-bp internal deletion). **Lane 6,** crude extracts from teratoma material harbouring the plasmid pFHEI714Δ4CR (39-bp promoter fragment).

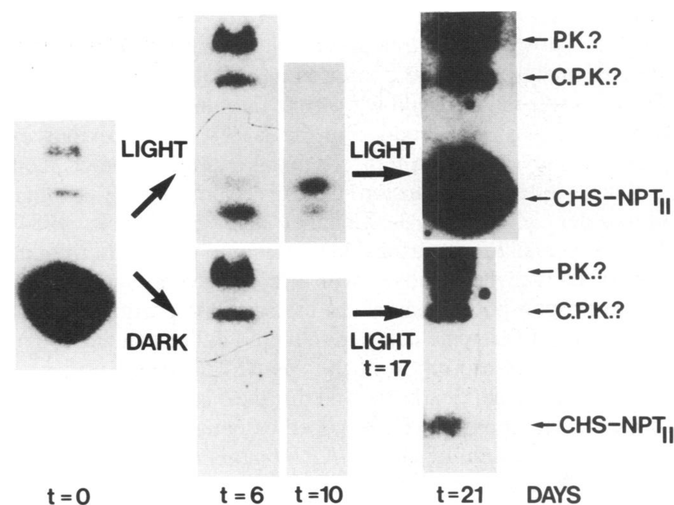

Fig. 7. Influence of light on the expression of the *chs-NPTII* gene in teratoma material. A teratoma callus containing the gene FHEI714CR (1.2-kbp promoter) was divided, one half was kept under the same light conditions as during the time of culture (16 h white light, 8 h dark), the other half was kept under continuous darkness. 17 days after starting the experiment the piece kept in the dark was illuminated again. At the times indicated (0, 6, 10 and 21 days) the NPTII activity was determined. This callus showed a very high variability in the absolute level of expression, but the qualitative difference between dark- and light-grown material is still apparent.

Influence of light on expression and transcription of the chs-NPTII gene in tobacco

To test whether light can regulate expression of the *chs-NPTII* genes, expression in light versus dark was compared. A teratoma callus containing the gene FHEI714CR (1.2-kbp promoter) was divided and either kept under normal light conditions as before (16 h white light, 8 h dark) or kept in continuous darkness. Protein was extracted from the teratoma material and the NPTII activity was tested. The results showed that in illuminated material the NPTII activity was present, whilst it was absent in dark-grown cells. The neomycin phosphotransferase activity could be restored if the etiolated cells were subsequently illuminated again (Figure 7). It should be mentioned, however, that in some transformants we were not able to switch off completely the expression in the dark or in some other transformants to switch it on again after a dark period. It could be that this was due to an unfavourable developmental stage of the callus or to an interfering effect of cytokinins on the regulation of expression. The results demonstrated that teratoma tissue might not be an ideal material to study light regulation of expression of the *chs-NPTII* gene. It has been shown that the flavonoid genes can be induced in seedlings only after a certain stage of differentiation. In seedlings from *Sinapis alba* the chalcone synthase becomes inducible ~30 h after germination (Steinitz *et al.*, 1976; Schäfer and Haupt, 1983). From this time on, the gene is competent for induction of expression by light in specific plant cells. Therefore, the best material to study expression of the *chs* gene are young plants grown under conditions where the flavonoid genes are not expressed, but competent for induction. Young tobacco plants grown in the absence of u.v.-B light should be ideal to study induction of transcription of the chimeric genes. We used the F1 generation of plant C containing the 1.2-kbp promoter construct for our experiments. Transformed plants were kept u.v. free in a plexiglass container where they developed normally and formed intact chloroplasts, up to an age of 4–6 weeks. Then they were illuminated with u.v.-B light. The mRNA of the neomycin phosphotransferase appeared 20 h after onset of illumination. This was shown by dot hybridization using the NPTII gene as a probe. The results also show that the *chs-NPTII* gene containing the 1.2-kbp promoter was not expressed in plants which were not induced by u.v.-B light. The concentration of the mRNA reached a maximum ~20–25 h after illumination (Figure 8). 45 h after onset of illumination the mRNA was degraded and not detectable by dot hybridization (data not shown). Looking under the same experimental conditions for the transcription of the endogenous small subunit of ribulose-1,5-bisphosphate carboxylase with a *rbcS*-cDNA clone from potato as a probe (kindly provided by L. Willmitzer; Eckes *et al.*, 1985), this gene was found to be transcribed both in u.v.-irradiated and in non-u.v.-irradiated plants (data not shown). These results indicate that transcription of *rbcS* is u.v.-B independent and must be regulated by light of a different quality, probably using other photoreceptors. The deletion mutants FHEI714Δ1CR, FHEI714Δ2CR, FHEI714Δ3CR and FHEI714Δ4CR have also been integrated in tobacco plants. Experiments are in progress to test the regulation of transcription of these genes.

Discussion

We have started some experiments which might help to define the molecular mechanism surrounding light-regulated expression of plant genes and which might lead to an understanding of the mechanism by which phytochrome can cooperate during induction of transcription with cryptochrome and the u.v.-B photoreceptor.

The promoter of the chalcone synthase gene from *A. majus* (3.9 kbp) was used for these experiments and ligated in front

of the NPTII gene from Tn5 (Beck *et al.*, 1982; Reiss *et al.*, 1984a). Various 5′ upstream deletion mutants have been constructed and all genes were inserted into tobacco teratoma cells. The cells were illuminated with white light in sterile culture and expression was measured using the NPTII test. Previous experiments have shown a transcriptional control of the chalcone synthase in cell suspension cultures from parsley to light (Kreuzaler *et al.*, 1983; Chappell and Hahlbrock, 1984). Therefore, making a reasonable assumption that transcriptional control or enzyme turnover will affect chalcone synthase and neomycin phosphotransferase to the same proportional extent, the amounts of enzyme detected in the NPTII test should mirror the extent of transcription of the *chs-NPTII* gene.

The experiments clearly showed that the 5′ upstream sequences of the *A. majus chs* gene are indeed essential but sufficient for maximal expression of the *chs-NPTII* gene. The construct with the 3.9-kbp promoter is expressed well in teratoma cells but if the promoter region is shortened to −39, leaving only 5 bp upstream from the TATA box intact, no expression of the *chs-NPTII* gene was observed.

We would like to mention that we obtained, after kanamycin selection (75 μg/ml), a few transformed teratoma calli with the 39-bp promoter construct (FHEI714Δ4CR) which were able to express a very reduced level of NPTII activity (5−10% compared with the 100% activity of the 1.2-kbp promoter construct). We believe that in these transformants, rearrangements had occurred which allowed them to survive the kanamycin selection. Similar results can be obtained with NPTII constructs without a promoter. With a low transformation rate kanamycin-resistant calli appear (J. Velten and A. Depicker, personal communication).

For the *chs-NPTII* genes the highest rate of expression was observed with the 1.2-kbp promoter construct. The 3.9-kbp promoter, which gave 80% expression, may contain some sequences upstream from position −1200, which repress the expression of the chimeric gene in a non-homologous host.

From these data we concluded that the chalcone synthase promoter which allows maximal expression of the chimeric gene in a non-homologous host has a size of ~1.2 kbp. This agrees with the observation of Morelli *et al.* (1985) who showed that a 5′ upstream region of 1.052 kbp is sufficient for expression of the ribulose-1,5-bisphosphate carboxylase small subunit from pea (*rbcS-E9*). These authors also showed that sequences 5′ of the TATA box, between positions −352 and −1052 are essential for optimal expression of the *rbcS-E9* gene.

The same observation has been made with the chalcone synthase promoter. Deletion of the sequences between −1200 and −357 decreased expression by a factor of 5. Another very important DNA sequence for gene expression is apparently located between positions −39 and −357. Deletion of this 318-bp fragment from the 1.2-kbp promoter (FHEI714Δ3CR) decreased expression by a factor of 4. Deletion of the sequence between −39 and −197 decreased the expression by a factor of 2.5 (FHEI714Δ1CR). This result is in contrast to the observation made with the promoter of the *rbcS-E9* gene from pea (Morelli *et al.*, 1985), in which sequences between −352 and −35 have little effect on the relative level of transcription. Morelli *et al.* identified a 33-bp sequence fragment (from −35 to −2) including the TATA box which would be involved in the light-regulated transcription of the *rbcS-E9* gene. Examination of this *rbcS-E9* light-responsive sequence revealed an 8-bp direct repeat and an imperfect palindrome. The direct repeat is homologous to other *rbcS* promoters (Morelli *et al.*, 1985). The *chs* promoter between −35 and −2 has only a 5-bp homology of CTTTG to the light-responsive sequence of the *rbcS-E9* promoter of pea.

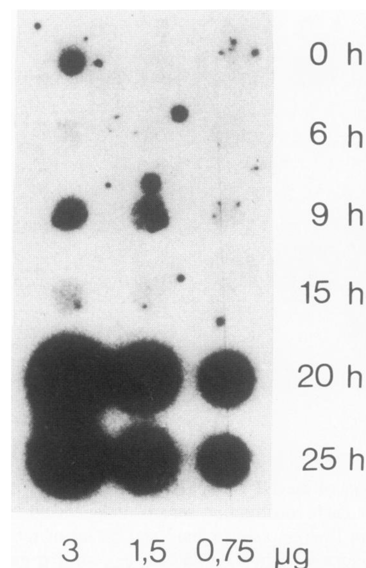

Fig. 8. Kinetics of induction for the *chs-NPTII* mRNA after onset of continuous u.v.-B illumination. The Fl progeny of plant C containing the 1.2-kbp promoter was used. 4-Week old transformed seedlings were kept u.v. free in a plexiglass container. After initiation of continuous u.v.-B illumination, ~2 g of leaf material was harvested at the indicated times (0, 6, 9, 15, 20 and 25 h). Total RNA was isolated and a dot blot hybridization was carried out as described in Materials and methods. The NPTII-coding region was used as a probe. For each time point different amounts of total RNA were spotted (3, 1.5 and 0.75 μg).

However, the 39-bp promoter construct (FHEI714Δ4CR) including this homology gave no expression of the *chs-NPTII* gene. We believe that the *chs* promoter contains light-responsive sequences different from those of the *rbcS-E9* promoter of pea and that all these sequences are located 5′ of the TATA box. That *chs* and Rubisco show different light induction behaviours in plants has been shown previously (Tobin and Silverthorne, 1985). This is supported by our finding that the endogenous *rbcS* gene in transformed tobacco plants is not induced by u.v.-B light, whereas induction of the *chs-NPTII* gene requires u.v.-B light.

Detailed analysis of the *chs* promoter of *A. majus* showed some remarkable sequences located within the 5′ upstream region (−39 to −197; −197 to −357; −357 to −1200; Sommer and Saedler, 1986). Three base pairs upstream from the TATA box (−38 to −44) the sequence TACCAT is present twice, separated by only 6 bp (−50 to −56) (Sommer and Saedler, 1986). Such a repeated sequence close to the transcriptional start site might be of importance for regulation of gene expression. Between positions −197 and −357 a long stretch of 33 As and Ts is located, just interrupted by a single G [TATAATATATTTTTTTTTT-ATTTAT (G) AATAAA −270 to −303].

The most striking structure of the *chs* promoter is found at −564 to −661. It is a 47-bp duplication separated by 3 bp, with ~85% homology between the repeats. Remarkably these repeats include a 'box' (GTGGTTAG) which is identical to the consensus core sequence for enhancers in animal genes (Gluzman and Schenk, 1983). The enhancer sequence includes the 3 bp separating the repeats and is itself not repeated. It is not yet clear whether the sequences mentioned above are really responsible for gene expression. Their influence will be proven in detail by integrating the sequences into a weak constitutive promoter and

measuring the level of transcription of these genes (D. Wing, in preparation).

Expression of the chimeric *chs-NPTII* gene containing the 1.2-kbp promoter seems to be light inducible in tobacco teratoma cells. This was demonstrated in a number of experiments where one half of a transformed callus was illuminated while the other portion was kept in the dark. All analysed transformants showed either a pronounced decrease of NPTII activity in the dark or a complete loss. We believe that the remaining NPTII activity of some transformants kept in darkness is due to an interfering effect of cytokinins.

Teratoma tissue transformed with the Ti plasmid pGV3851 still contain the gene 4 from the T-DNA, which is involved in the biosynthesis of cytokinins. It has been shown that cytokinins can induce and enhance expression of the flavonoid genes in various plants (McClure, 1975). The best system therefore to study regulation of transcription might be young seedlings or young plants grown without u.v.-B light. We used the F1 generation of plant C containing the 1.2-kbp promoter construct to produce seedlings hopefully competent for u.v.-B light induction.

The result clearly demonstrated (Figure 8) that transcription of the *chs-NPTII* gene was inducible by u.v.-B light and regulated by the 5′ upstream sequences in a meaningful way, even in a non-homologous host. In young plants shielded from u.v.-B light, no NPTII mRNA can be demonstrated. Upon illumination of such transformed plants with u.v.-B light, the concentration of the *NPTII* mRNA reached a maximum after ~20 h. The newly synthesized mRNA was degraded after 45 h.

The curve of *chs-NPTII* gene transcription is very similar to that observed with the intact *chs* gene in cell suspension cultures from parsley. In these cells, the maximum *chs* mRNA concentration is reached 10 – 12 h after onset of illumination (Kreuzaler *et al.*, 1983).

This is, to our knowledge, the first demonstration that transcription of a chimeric gene integrated into the genome of a non-homologous host can be switched on and studied under natural conditions.

In addition, we have shown that the induction of the *chs-NPTII* gene is primarily dependent on the action of a u.v.-B photoreceptor which presumably mediates the signal to the DNA. Conditions which lead to normal chloroplast development in the transformed plants were not able to switch on the *chs-NPTII* gene transcription.

The sequence deletion mutants and regulatory behaviours of the *chs* promoter demonstrate that the *chs* gene is unlike the *rbcS* small subunit gene of pea and tobacco. We hope to eventually determine which promoter sequences interact in the u.v.-B induction pathway and unravel the different promoter structures plants have evolved in response to the different light signals they receive.

Materials and methods

Strains and plasmids

E. coli: DHI (F−, rec A1, end A1, sup E44, gyr A96), GJ23 (R64drd11, pGJ28, SmR, TcR, KmR). *A. tumefaciens:* C58Cl (pGV3850, RifR, CbR), C58Cl (pGV3851, RifR, CbR).

Conjugation between *E. coli* and *A. tumefaciens* was done by using the strain GJ23 containing the helper plasmids R64drd11 and pGJ28 (Van Haute *et al.*, 1983). *A. tumefaciens* strain C58Cl containing either the Ti plasmid pGV3850 or pGV3851 were used for transformation of *Nicotiana tabacum* Wiconsin 38.

Recombinant DNA techniques

Analysis of plasmid DNA (mini prep) was done according to Birnboim and Doly (1979). For large-scale preparations of DNA, the method of Maniatis *et al.* (1982) was used. Purification, electrophoresis and hybridization analysis of total *A. tumefaciens* DNA was done according to Dhaese *et al.* (1979).

Media and culture

Bacterial media used were Luria broth (LB), DYT (Maniatis *et al.*, 1982) and Minimal A (Min A) media from Miller (1972).

The concentration of antibiotics used was as follows: *E. coli*: ampicillin; Amp (100 μg/ml), spectinomycin; Sp (100 μg/ml), tetracycline; Tet (12.5 μg/ml), kanamycin; Km (50 μg/ml). *A. tumefaciens:* carbenicillin; Cb (100 μg/ml), rifampicin; Rif (100 μg/ml).

Plant media used were Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Linsmaier and Skoog (LS) (Linsmaier and Skoog, 1965).

Nopaline assay

The presence or synthesis of nopaline due to the expression of the *nos* gene in transformed calli and regenerating shoots was monitored according to Otten (1982).

Plant transformation

Small axenically growing plants were kept in LS medium in jars and were decapitated and inoculated with *Agrobacterium* strains. When the strain C58Cl pGV3851 was used, wound calli were removed after 3 weeks and placed on hormone-free LS medium with 500 μg/ml cefotaxime (Hoechst). Teratoma calli developed and when kanamycin selection was used, 50 – 250 μg/ml kanamycin was added. The strain C58Cl pGV3850 was used in a co-infection procedure with the octopine shooter strain GV2215 (GV3850:GV2215 mixed in a ratio 5:1). Calli containing shoots were removed from the wound surface and put on hormone-free LS medium with 500 μg/ml cefotaxime. Emerging shoots were tested for nopaline production. Nopaline-positive shoots were propagated on MS medium. When these shoots reached a sufficient size they were transferred to soil and grown in the greenhouse.

Isolation of RNA and RNA dot blots

Plant material frozen in liquid nitrogen was homogenized using a pestle and mortar in a guanidinium hydrochloride buffer (8 M guanidinium hydrochloride; 20 mM MES pH 7.0; 20 mM EDTA; 50 mM β-mercaptoethanol). The homogenate was filtered through Miracloth (Calbiochem) and centrifuged for 10 min at 500 *g* to remove the debris. The supernatant containing the RNA was precipitated by adding 1/20 vol. 1 M acetic acid and 0.7 vol. ethanol. The pellet was washed twice with 70% ethanol and dissolved in H_2O. One vol. of 2x extraction buffer [2% cetyltrimethylammoniumbromide (CTAB) (w/v), 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl] was added and the aqueous phase was extracted twice with chloroform:isoamylalcohol (24:1). The RNA was precipitated adding 2 vol. of precipitation buffer [1%CTAB (w/v); 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% β-mercaptoethanol]. The RNA was resuspended in 200 – 400 μl of 1 M CsCl solution (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 50 mM NaCl, 1.0 M CsCl) and precipitated again with 2 vol. of ethanol. RNA was denatured with formaldehyde and dot blots were performed as described by White and Bancroft (1982). Hybridizations were carried out as described by Willmitzer *et al.* (1982).

Analysis of chs by immunoblotting

Crude protein extracts were obtained by homogenization of plant material in an equal volume of buffer [w/v; glycerol 30%, 2.1 M β-mercaptoethanol, SDS 7.2%, Tris 185 mM, 0.5 ml bromophenol blue (0.1%); pH 6.8]. Aprotinin (trade name Trasylol), a protease inhibitor, was used at a final concentration of 10 μg/150 μl.

About 30 μg of total protein were boiled for 5 min and loaded on a slab gel according to Laemmli (1970) with 12% acrylamide. Marker proteins were α-macroglobulin (170 000), phosphorylase b (97 400), glutamate dehydrogenase (55 400), lactate dehydrogenase (36 500); trypsin inhibitor (20 100) (Combithek, Boehringer). The electrophoretic transfer was done at 4°C in transfer buffer (0.025 M Tris; 0.192 M glycine; 20% v/v methanol) according to Towbin *et al.* (1979).

The quality of transfer was monitored by staining with Ponceau red. Immunoblots were treated with affinity-purified anti-*chs* at 7.5 μg/ml for 2 h at room temperature followed by peroxidase-labelled sheep anti-rabbit antibodies (Institut Pasteur) diluted 1:200 for the same amount of time.

Neomycin phosphotransferase assay

The *in situ* gel assay for NPTII activity developed by Reiss *et al.* (1984b) was used with slight modifications for plant tissue, as described by Schreier *et al.* (1985). To minimize the noted variability in the NPTII levels of individual calli transformed with the same chimeric NPTII gene, pools of 10 calli were used to compare relative promoter strength. Protein concentration was determined by the method of Bradford (1976).

Transient expression of protoplasts

Protoplasts were kept in co-culture with *Agrobacterium* according to Marton *et al.* (1979).

An overnight culture of the *Agrobacteria* strain C58Cl (pGV3850::FHEI714CR) and the control C58Cl (pGV3850::1103neo) (Hain *et al.*, 1985) were each add-

ed to the 3-day old protoplasts over a period of 24 h. Samples were taken from each co-culture over a period of 7 days. After pelleting 1.2×10^5 initial protoplasts with sea water (600 mOsm; 500 *g*; 10 min) they were transferred to Eppendorf tubes. A quick spin made it possible to remove any remaining fluid. After addition of 17 μl glycerol (90%) and 12.5 μl 4-times concentrated NPTII extraction buffer without SDS (buffer described by Schreier *et al.*, 1985) protoplasts were kept at −70°C. Before loading the samples onto the non-denaturing polyacrylamide gel, SDS and water was added until a normal concentration of 1x NPTII extraction buffer was reached.

Induction of transformed tobacco plants with u.v. light

Transformed tobacco plants were grown u.v.-B (290−350 nm) free in a plexiglass container in the greenhouse. After ~4 weeks the normal looking plants were illuminated with continuous light (3x Osram L, 40 W15; 1x Osram L 40 W/73; 1x Philips TL 40 W 21). During illumination the plants were shielded with a glass filter, which absorbs u.v.-light of wavelengths <310 nm (Optisches Farbglas WG 305 230x70 MM, Firma Schott, Mainz). Wavelengths <305 nm damage tobacco cells, so that no induction of the endogenous chalcone synthase can be seen (E. Wellman, personal communication).

Acknowledgements

We wish to thank I. Czaja and E. Theilenberg for excellent technical assistance. We are indebted to H. Sommer for providing the genomic clone of the *A. majus chs* gene and its sequence before publication; to E. Wellmann for help and advice in developing a *chs* induction system for tobacco, to G. and G. Neuhaus for injecting the construction into *Acetabularia* and to E. Goyvaerts and P. Czernilofsky for support during the transient expression assay. We also want to thank D. Wing for stimulating discussion and critical reading of the manuscript and J. Freitag for typing the manuscript. The help of our colleagues from the Laboratories of Genetics in Gent and Brussels is gratefully acknowledged. H.K. was supported by a pre-doctoral fellowship from the Max Planck Society.

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Received on 4 November 1985