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A promoter region that controls basal and elicitor-inducible expression levels of the NADPH:cytochrome P450 reductase gene (*Cpr*) from *Catharanthus roseus* binds nuclear factor GT-1

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Abstract NADPH:cytochrome P450 reductase (CPR) is essential for the activation of cytochrome P450 enzymes, which are involved in a wide variety of metabolic pathways in plants, including those related to defence responses. In the subtropical plant *Catharanthus roseus* several cytochrome P450 enzymes operate in the biosynthesis of defence-related terpenoid indole alkaloids (TIAs). In agreement with the importance of CPR in defence, *Cpr* mRNA levels in *C. roseus* were found to be enhanced by fungal elicitor preparations that also induce TIA biosynthesis and P450 gene expression. Here we describe the isolation of a *C. roseus* genomic DNA clone covering the 5′ part of the *Cpr* gene and 1.6-kb of upstream sequences. Mapping of the transcription start site showed the untranslated leader sequence is approximately 280 bp long. To study the control of gene expression by the *Cpr* promoter, transcriptional fusions between *Cpr* promoter fragments and the *gusA* reporter gene were generated and their expression was analyzed in stably transformed tobacco plants. The *Cpr* promoter fragment extending from −1510 to −8, with respect to the ATG start codon, conferred basal and elicitor-inducible expression on the *gusA* reporter gene, strongly indicating that the *Cpr* gene of *C. roseus* is indeed controlled by this promoter region. Progressive deletion from the 5′ end of the promoter to position −632 had little effect on *gusA* expression. However, deletion to position −366 resulted in a complete loss of basal activity and largely eliminated elicitor-induced expression, indicating that the region from −632 to −366 contains the main transcription-enhancing *cis*-regulatory sequences. Electrophoretic mobility shift assays with tobacco nuclear extracts showed that binding sites for nuclear factor GT-1 are redundant in the *Cpr* promoter, but absent from the downstream part of the leader sequence. The presence of strong GT-1 binding sites in the main enhancer region (−632 to −366), is suggestive of a functional role for this factor in basal expression and elicitor responsiveness of the *Cpr* promoter.

Key words *Catharanthus roseus* · NADPH:cytochrome P450 reductase · *Cpr* · Elicitor · GT-1

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Introduction

Cytochrome P450 monooxygenases occupy a central position in the oxidative metabolism of plants, animals and microorganisms. In plants, these enzymes are involved in numerous biosynthetic pathways, including those leading to lignins, terpenoids, sterols, fatty acids, hormones, pigments and defence-related phytoalexins (reviewed by Schuler 1996). Furthermore, certain plant cytochrome P450 enzymes are capable of detoxifying foreign compounds, such as herbicides (reviewed by Schuler 1996). All cytochrome P450 monooxygenases are dependent for their activity on reducing equivalents. In most cases NADPH serves as an electron donor and electron transfer is mediated by the flavoprotein NADPH:cytochrome P450 reductase (CPR; E.C. 1.6.2.4) (Lu et al. 1969). *Cpr* genes have been cloned from various organisms, and domains involved in cofactor and substrate binding were shown to be highly conserved among all known sequences (Shen and Kasper 1993). We previously isolated a *Cpr* cDNA clone from the subtropical plant *Catharanthus roseus* (Madagascar periwinkle) by immunoscreening of a cDNA

expression library with antiserum raised against the purified CPR protein (Meijer et al. 1993a). Southern blot analysis indicated that *Cpr* occurs as a single-copy gene in the *C. roseus* genome. A single *Cpr* gene was also identified in *Vicia radiata*, but some other plants (*Arabidopsis thaliana*, *Helianthus tuberosis*) were shown to contain two *Cpr* copies (Lesot et al. 1995). Although we cannot completely exclude the possibility that the *C. roseus* genome contains a second, less conserved *Cpr* gene copy, not detected by hybridization with our cDNA clone, it seems most likely that all *C. roseus* cytochrome P450 enzymes are dependent on a single form of NADPH:cytochrome P450 reductase, as in animals and fungi. As yet the physiological significance of multiple *Cpr* genes in some other plants remains to be elucidated (Lesot et al. 1995).

C. roseus is a valuable source of a number of terpenoid indole alkaloids (TIAs) that find use in the medical treatment of circulatory diseases and cancer. TIAs are composed of an indole moiety, provided by the amino acid derivative tryptamine, and a terpenoid moiety, provided by the secoiridoid secologanin. In plants, this group of secondary metabolites may have antifeedant activity (Aerts et al. 1991) or may function in pathogen defence (Luijendijk 1995). Progress in the understanding of TIA biosynthesis may eventually provide ways to improve product yield by genetic modification of plants or cell cultures. Consequently, the regulation of enzymes and genes involved in TIA biosynthesis has become the subject of extensive research efforts (reviewed by Meijer et al. 1993b; Hashimoto and Yamada 1994; Kutchan 1995). At least two cytochrome P450 monooxygenases have been shown to be involved in TIA biosynthesis in *C. roseus*. One of these is geraniol 10-hydroxylase (monoterpene hydroxylase), which catalyses an early step in secologanin biosynthesis (Meehan and Coscia 1973; Meijer et al. 1993c). The second is tabersonine 16-hydroxylase, which operates in the biosynthesis of the TIA vindoline (St-Pierre and De Luca 1995). Cytochrome P450 is also involved in the phenylpropanoid pathway, which, like the TIA pathway, plays an important role in plant defence metabolism (Schuler 1996). In *C. roseus*, the *Cpr* mRNA level is enhanced by fungal elicitor preparations (Meijer et al. 1993a), which are known to induce cytochrome P450-dependent defence-related biosynthetic pathways, including TIA biosynthesis (Moreno et al. 1995; Schuler 1996). Hence, besides being required for a number of pathways in primary metabolism, *Cpr* may also be of key importance in the regulation of plant defence metabolism. Elicitor induction of *Cpr* was shown to occur co-ordinately with induction of two other genes known to function in TIA biosynthesis, the tryptophan decarboxylase (*Tdc*) and strictosidine synthase (*Str1*, previously termed *Sss*) genes (Pasquali et al. 1992; Meijer et al. 1993a). Therefore it is possible that in *C. roseus* the *Cpr*, *Tdc* and *Str1* gene promoters interact with the same nuclear factors.

Here we describe the isolation of a genomic DNA clone covering the 5′ part of the *Cpr* gene and 1.6 kb of upstream sequences. As a first step towards understanding *Cpr* regulation, we have studied basal and elicitor-induced expression of transcriptional fusions between *Cpr* promoter fragments and the *gusA* reporter gene in transgenic tobacco plants. Furthermore, we demonstrate that nuclear factor GT-1 interacts with the *Cpr* promoter in vitro.

Materials and methods

Northern blot analysis of elicitor-induced cell suspension cultures

C. roseus cell suspension cultures, elicitor preparation, elicitor treatment of cell suspension cultures, RNA isolation and Northern blot analysis have all been described in Meijer et al. (1993a). Northern blots were probed with the complete 2.3-kb insert of the *Cpr* cDNA clone pSK-R9 (Meijer et al. 1993a), or with 0.2-kb fragments corresponding to the 3′ non-coding regions of the *Cyp72A1* (P450 type II, Meijer 1993) and *Cyp72A1v2* (P450 type I, Meijer 1993) cDNAs. With these probes no cross-hybridization between *Cyp72A1* and *Cyp72A1v2* is detected.

Genomic library construction and screening

C. roseus total DNA, partially digested with *Sau*3AI, was cloned in λGEM11 (Promega) *Bam*HI arms (Goddijn et al. 1994). Library screening was performed as described in Memelink et al. (1994).

Nucleic acid sequencing

A 1.4-kb genomic *Hin*dIII-*Xho*I DNA fragment from a λGEM11 clone containing the promoter region of the *Cpr* gene was subcloned in pBluescript II SK+ (Stratagene). Progressive deletions from both ends of the promoter fragment were generated with the Erase-a-base system (Promega) and the resulting deletion constructs were sequenced according to the Sequenase method (US Biochemical). The 5′ end of the *Cpr* mRNA was directly sequenced by primer extension as described by Geliebter (1987) using 10 μg of poly(A)+ RNA and the oligonucleotide 5′-CGGCGACAA-CTTCTCCG-3′. The poly(A)+ RNA was obtained from *C. roseus* suspension culture cells and isolated with a Poly(A) Tract kit (Promega).

Vector construction and generation of transgenic plants

A deletion series of *Cpr* promoter fragments were fused to the *gusA* gene in the GusXX vector described by Pasquali et al. (1994), resulting in constructs CprΔ1510, CprΔ817, CprΔ632 and CprΔ366 (see Fig. 4A). To obtain construct Cpr-47CaMV, the −1510 to −366 *Cpr* region was cloned in vector GusXX-47 (Pasquali et al. 1994), upstream of a truncated (−47) cauliflower mosaic virus (CaMV) 35S promoter fused to the *gusA* gene. Subsequently the *Cpr-gusA* fusion genes were cloned in the binary vector pMOGλCAT (Pasquali et al. 1994). The binary vector constructs were introduced into *Agrobacterium tumefaciens* LBA 4404 and the resulting bacterial strains were used to inoculate leaf discs of *Nicotiana tabacum* cv. Petit Havanna SR1 according to Horsch et al. (1991). Transgenic plants were selected with 100 μg/ml of each of the antibiotics kanamycin, vancomycin and cefotaxime on solidified MS medium (Murashige and Skoog 1962) containing 3% sucrose. Growth conditions were 21° C and 16 h light (2000 lux) per day.

Preparation of leaf extracts and β-glucuronidase (GUS) enzyme assays

Leaf discs (5 mm diameter) were prepared from 6- to 8-week-old transgenic tobacco plants grown in tissue culture jars, and were incubated for 24 h in water or in an autoclaved solution of 1.5% yeast extract (Difco). Proteins were prepared by homogenization of the leaf discs with a Potter S homogenizer (Braun) in GUS extraction buffer. GUS enzyme activities were measured according to Jefferson (1987) using a Perkin-Elmer LS5 fluorimeter and 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate. Protein concentrations were determined according to Bradford (1976).

Electrophoretic mobility shift assays (EMSAs)

Nuclear protein extracts were prepared from mature tobacco leaves as described by Green et al. (1991). EMSA reactions contained 0.1 ng of ^{32}P-end- labelled probe, 3 μg of poly(dIdC) · poly(dIdC) and variable amounts of protein and competitor DNAs (as indicated in the Figures) in 10 μl of nuclear extraction buffer (Green et al. 1991). The reactions were incubated for 20 min at room temperature and subsequently loaded under an applied voltage (10 V/cm) on 5% acrylamide/bisacrylamide (37.5:1) gels in 0.5 × TRIS-borate/EDTA (45 mM TRIS-borate, 1 mM EDTA pH 8.3) buffer. Gels were dried on Whatman DE81 paper and autoradiographed.

Results and discussion

Elicitor induction of *Cpr* and P450 gene expression

Fungal elicitor preparations are known to induce the expression of various defence-related biosynthetic pathways in plants, many of which are dependent on cytochrome P450 activities and, consequently, also on the activity of CPR (Schuler 1996). In *C. roseus* the cytochrome P450-dependent biosynthesis of TIAs, as well as phenolics, was found to be induced by fungal elicitors (reviewed by Moreno et al. 1995). Previously we showed that *Cpr* mRNA levels in *C. roseus* cell suspension cultures could be enhanced by treatment with either of two fungal elicitor preparations, yeast extract and *Pythium aphanidermatum* culture filtrate (Meijer et al. 1993a). Here we demonstrate that the same elicitor preparations also induce expression of at least two cytochrome P450 genes, *Cyp72A1* and *Cyp72A1v2*, described in Meijer (1993) and Vetter et al. (1992). The precise function of these genes is unknown, but because of the abundance of their products in cell cultures, they are thought to function in a major secondary metabolic pathway, such as TIA biosynthesis (Vetter et al. 1992). As shown in Fig. 1, addition of either yeast or *Pythium* elicitor resulted in a rapid induction of both *Cpr* and *Cyp72A1* expression. The induction profile of *Cyp72A1v1* was identical to that of *Cyp72A1* (data not shown). The *Cpr* mRNA level was already at its maximum after 1 h and started to decline after 8 h. *Cyp72A1* showed a slower time course of induction, with maximum levels being attained between 4 and 8 h. A similar difference in the time course of induction of cytochrome P450 and CPR was observed in rat following treatment with phenobarbital (Hardwick et al. 1983). A possible explanation

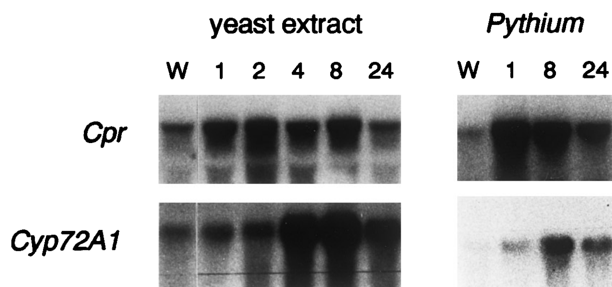

Fig. 1 Induction by elicitor of *Cpr* and cytochrome P450 gene expression. *C. roseus* cell suspension cultures were exposed for 1 to 24 h to yeast extract or *Pythium aphanidermatum* culture filtrate, as indicated at the top. Control cultures were incubated for 24 h in water (W). Northern blots containing total RNA (10 μg/lane) were probed with the *Cpr* or *Cyp72A1* cDNAs

for the different time courses is that the signal transduction pathways leading to cytochrome P450 and *Cpr* induction may involve different components. Alternatively, a common transcription factor activated by the elicitor signal may possess different affinities for the cytochrome P450 and *Cpr* gene promoters. The observation that elicitors induce both *Cpr* and cytochrome P450 gene expression, suggests that enhanced CPR enzyme activity is required for optimal activity of the induced cytochrome P450 enzymes. This is also likely to be the case in the mammalian liver, since several cytochrome P450 inducers have been shown to induce *Cpr* expression or CPR activity as well (Hardwick et al. 1983; Simmons et al. 1987). Another observation that strongly suggests that CPR can be limiting for cytochrome P450 activity in plants, is that transformation of tobacco with a gene construct encoding a fusion protein between rat cytochrome P4501A1 and yeast CPR could confer herbicide resistance, whereas transformation with a construct encoding the cytochrome P4501A1 alone was ineffective (Shiota et al. 1994). The apparent importance of *Cpr* induction for increased cytochrome P450 activities prompted us to isolate the *Cpr* gene promoter for further studies of *Cpr* gene regulation.

Isolation and sequence analysis of the *Cpr* gene promoter

A genomic DNA library of *C. roseus* in λGEM11 was screened and a 4.5-kb *Xho*I fragment, which hybridized with the 5′ part of the *Cpr* cDNA (Meijer et al. 1993a), was isolated from a positive lambda clone. Southern blot analysis indicated that this fragment contains approximately 1.6 kb of upstream sequences, 0.7 kb of 5′ *Cpr* coding region, a 1.1-kb first intron, a second exon of 0.3 kb, and part of a second intron (Fig. 2A). Sequence analysis of a subfragment containing the first exon confirmed that the genomic DNA sequence corresponded to that of the *Cpr* cDNA (Meijer et al. 1993a). An *Xho*I-*Hin*dIII fragment containing the *Cpr* promoter region and part of the mRNA leader sequence was then

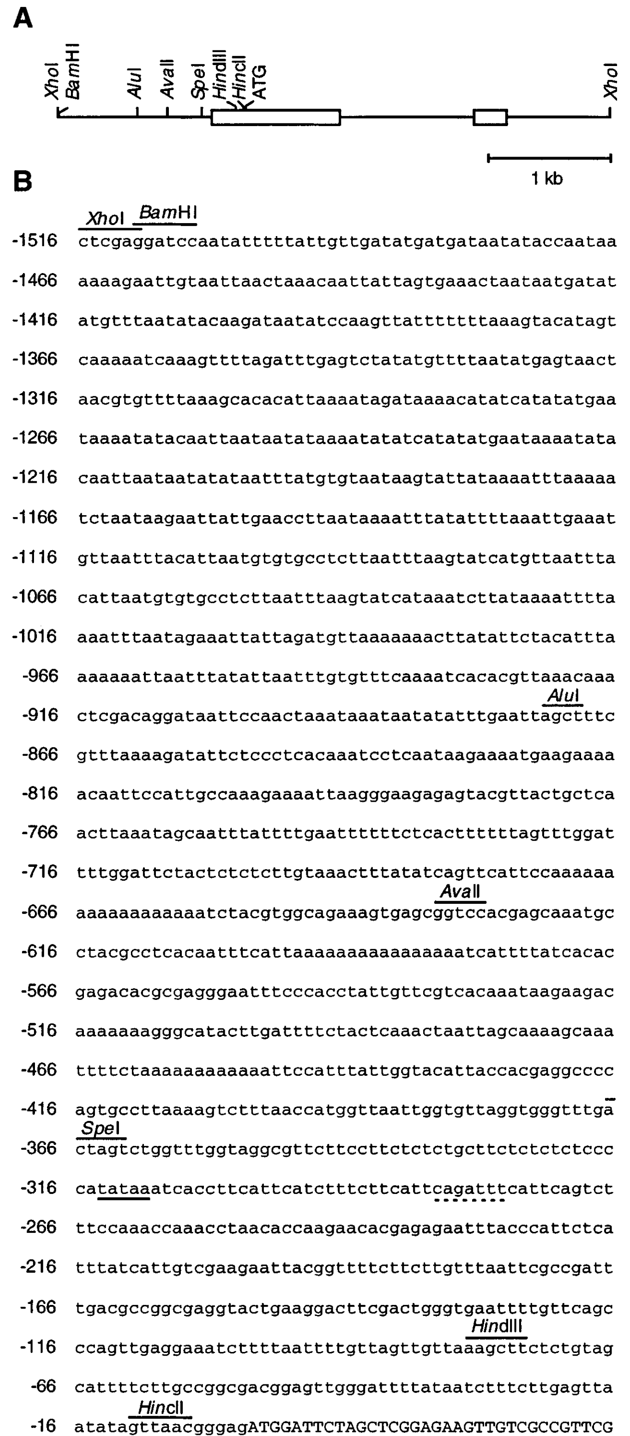

Fig. 2A, B Structure and nucleotide sequence of the 5′ part of the *Cpr* gene. **A** Map of a 4.5-kb genomic DNA fragment containing the promoter region and first two exons (indicated by the *open boxes*) of the *Cpr* gene. ATG indicates the position of the putative translation initiation codon. The 3′ part of the *Cpr* coding region is not located in this fragment. **B** Nucleotide sequence of the 5′ part of the *Cpr* gene. Coding sequences are shown in *upper case* and upstream sequences in *lower case letters*. The putative TATA box is indicated by the *solid underline* and the approximate location of the transcription start site is indicated by the *broken underline*. Restriction sites used in the construction of *Cpr-gusA* gene fusions are indicated in **A** and **B** and are not necessarily unique. The *Xho*I and *Bam*HI sites at the 5′ end of the nucleotide sequence are derived from the λGEM11 vector. The nucleotide sequence has been deposited in the EMBL, Genbank and DDBJ databanks under the accession number Y09417

subcloned and progressive deletions were generated to sequence the fragment on both strands. The nucleotide sequence of the *Cpr* promoter and part of the coding region is shown in Fig. 2B. Direct sequencing of *Cpr* mRNA indicated that the transcription start site is located around positions −276 to −283 relative to the first ATG codon (Fig. 3). A putative TATA box is located at position −314.

In vivo analysis of chimaeric *Cpr-gusA* fusion constructs

For functional characterization of the *Cpr* promoter, transcriptional fusions between *Cpr* promoter fragments and the *gusA* reporter gene were generated. As shown in Fig. 4A, construct CprΔ1510 contained the −1510 to −8 *Cpr* promoter sequence upstream of the *gusA* gene, and constructs CprΔ817, CprΔ632 and CprΔ366 contained *Cpr* promoter sequences truncated down to positions −817, −632 and −366, respectively. In vivo analysis of these constructs was performed in tobacco plants, because of the lack of an efficient transformation/regeneration procedure for *C. roseus*. For each construct more than ten independent transgenic tobacco plants were generated and analyzed for GUS activities. The results are presented in a scatter diagram (Fig. 4B) in which each symbol represents the GUS activity of a single plant. Analysis of the longest promoter construct (CprΔ1510) demonstrated that the *Cpr* promoter was capable of driving detectable expression of the *gusA* reporter gene in 9 out of 12 plants analyzed. In these 9 plants, GUS activities ranged from 0.06 to 2.0 nmol/min/mg protein. Variation between GUS activities of individual transformants is usually found in promoter studies and is probably due to differences in chromosomal position and/or copy number of the transgenes. To test whether the *Cpr* promoter could confer elicitor-inducible expression on the *gusA* reporter gene, leaf discs of the transgenic tobacco plants harbouring construct CprΔ1510 were incubated for 24 h in a solution of 1.5% of yeast extract prior to GUS activity measurements. All 9 plants that had detectable GUS activity without elicitor treatment (incubated for 24 h in water as a control), showed significantly elevated GUS activity levels after elicitor treatment (Fig. 4B). Tobacco plants harbouring constructs CprΔ817 and CprΔ632 showed similar GUS activities to the CprΔ1510 plants, both with and without elicitor treatment (Fig. 4B). In contrast, the activity levels of plants harbouring construct CprΔ366 were markedly reduced (Fig. 4B). Without elicitor treatment none of these plants showed detectable GUS activity and following elicitor treatment, in 4 out of 11 plants, the GUS activities were just above the detection limit. To

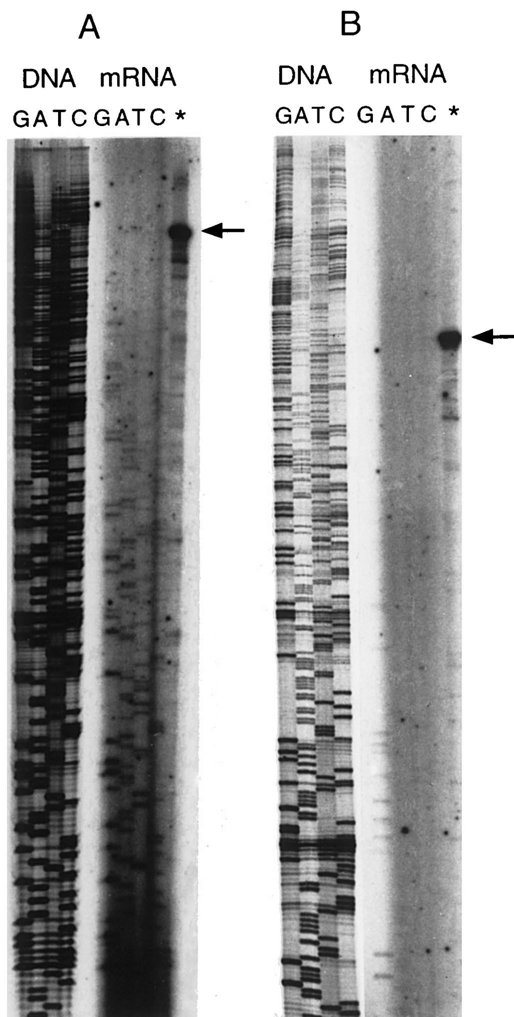

Fig. 3A, B Direct sequencing of *Cpr* mRNA by primer-extension. Panels **A** and **B** display, respectively, a long and short run of the sequencing reactions. For reference, in both panels a sequencing ladder of genomic *Cpr* DNA is shown next to the sequencing ladder of the *Cpr* mRNA. The lanes marked with *asterisks* display a primer extension reaction in the absence of dideoxynucleotides. The longest reaction product, corresponding to the transcription start site, is indicated by an *arrow*

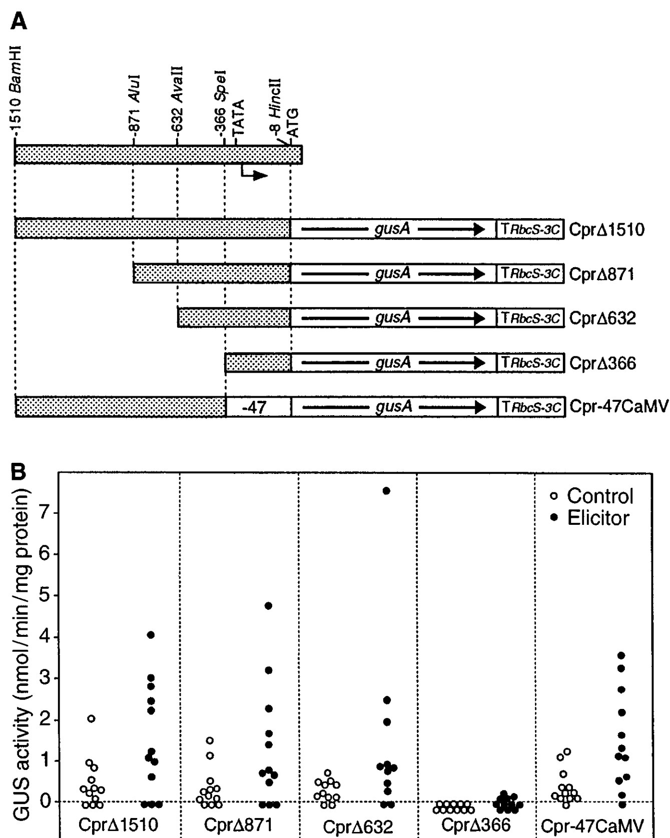

Fig. 4A, B Expression of chimaeric *Cpr-gusA* fusion constructs in transgenic tobacco plants. **A** Schematic representation of the *Cpr-gusA* constructs. A map of the *Cpr* promoter from −1516 to +50 is shown at the top. The *arrow* shows the position of the transcription start site. The TATA box, putatitive ATG start codon (at position +1) and restriction sites used for the construction of *Cpr-gusA* fusions are indicated. **B** Expression of the constructs CprΔ1510, CprΔ817, CprΔ632, CprΔ366 and Cpr-47CaMV in transgenic tobacco plants. For each construct, 11 or 12 independent transgenic plants were analyzed. GUS activities were measured in protein extracts prepared from leaf discs incubated in water (*open circles*) or in 1.5% yeast extract (*filled circles*). Symbols below zero level indicate that GUS activities were below the detection limit

further demonstrate the importance in *Cpr* regulation of the sequences upstream of −366, we tested whether this region was functional when cloned upstream of the truncated CaMV −47 35S promoter that is devoid of basal activity (data not shown). As shown in Fig. 4B, the −1510 to −366 region conferred basal as well as elicitor-inducible expression on the −47 promoter (construct Cpr-47CaMV), resulting in similar GUS activity levels to those observed for the constructs CprΔ1510, CprΔ817 and CprΔ632.

In conclusion, it appears that deletion of the *Cpr* promoter up to position −632 has little or no effect, which indicates that the upstream region does not contain *cis*-regulatory elements required for determining the basal expression level. An alternative possibility is that such elements do occur but are redundant in the *Cpr* promoter or function in other tissues or under different conditions. The region between −632 and −366 was shown to be required for detectable basal reporter gene activity, indicating that this region contains the main transcription-enhancing sequences. Furthermore, this region appears to confer elicitor responsiveness, as can be concluded from the observations that deletion of the −632 to −366 region largely eliminates elicitor induction and that the *Cpr* promoter region upstream of −366 can confer elicitor inducibility on an otherwise inactive heterologous promoter. In addition, a weak elicitor-responsive element may be located downstream of −366, since construct CprΔ366, which showed no detectable basal activity, was still slightly elicitor inducible in some plants.

Since the *Cpr* promoter was found to confer elicitor-inducible expression in tobacco, it is possible that the previously observed elicitor-inducible *Cpr* mRNA levels

in *C. roseus* (Meijer et al. 1993a) were also the result of a transcriptional induction of *Cpr* gene expression. Therefore, it seems likely that *C. roseus* and tobacco have a conserved mechanism that mediates elicitor induction of the *C. roseus Cpr* gene. The *Cpr* gene product is required for the activity of cytochrome P450 enzymes, which are known to operate in the biosynthesis of several compounds produced as a result of defence reactions in various plants. Thus, in view of the function of the *Cpr* gene, it is not unlikely that the signal transduction pathway leading to induction of the *Cpr-gusA* gene constructs will also induce the endogenous *Cpr* gene in tobacco and that this pathway is conserved in other plant species as well.

Characterization of in vitro binding of nuclear factors to the *Cpr* promoter

To identify *trans*-acting factors interacting with the *Cpr* promoter, eight subfragments, A to H (Fig. 5A), were used in electrophoretic mobility shift assays (EMSAs) with nuclear extracts prepared from mature tobacco leaves. A single complex of low mobility was observed in EMSAs with fragment E, which covers the main enhancer region (−632 to −366) identified during the in vivo analysis of *Cpr-gusA* fusion constructs (Fig. 5B). A low-mobility complex is characteristic of the binding of nuclear factor GT-1, which was previously identified as a factor that interacts with the box II sequence in the pea *RbcS-3A* promoter (Green et al. 1987). To test whether the fragment E shift was indeed due to GT-1 binding, competition assays were performed with a tetramer (4II) of the *RbcS-3A* box II sequence (5′-TGTGTGGTTA-ATATG-3′) and with two mutant versions of the box II tetramer, 4IIm1 (4 × 5′-TGTGTCCTTAATATG-3′) and 4IIm2 (4 × 5′-TGTGTGGGGAATATG-3′). The mutations of GG to CC, or TT to GG in box II were shown to eliminate binding of GT-1 completely and therefore serve as useful controls for specificity (Green et al. 1988). As

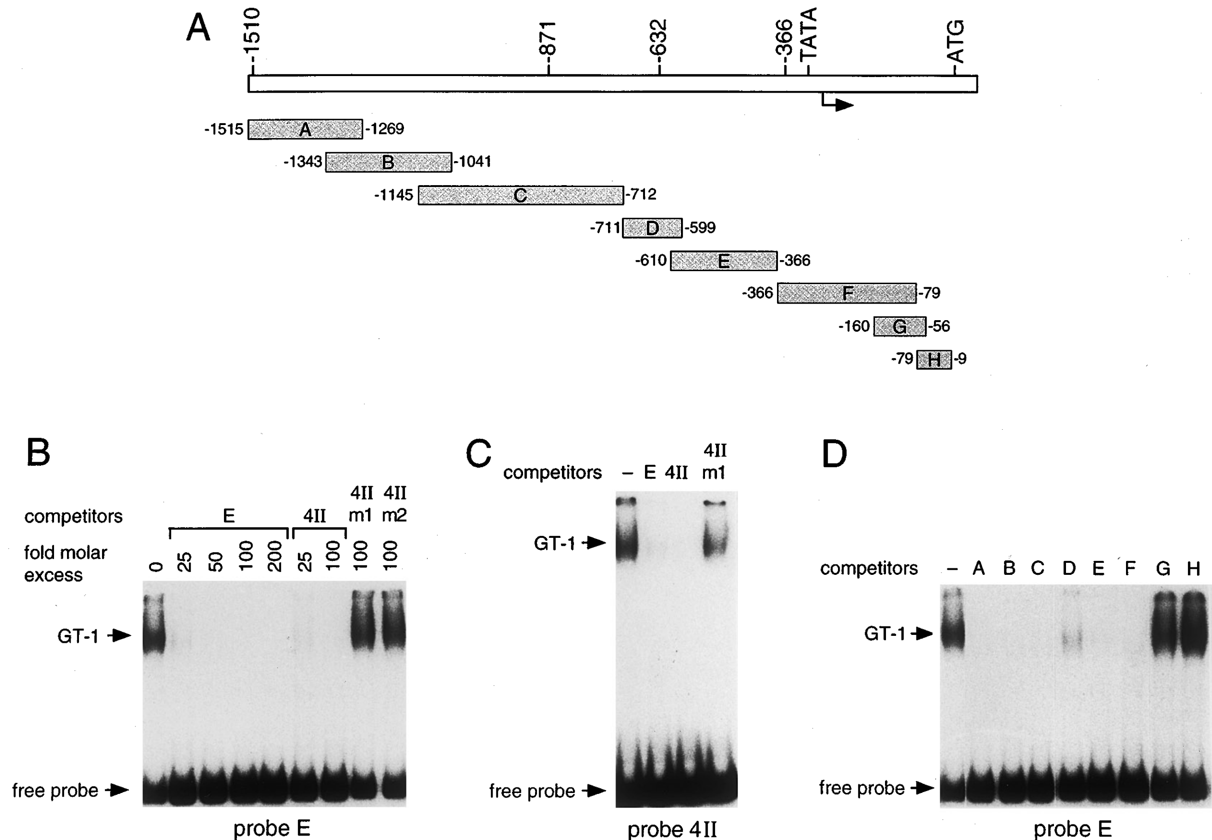

Fig. 5A–D Electrophoretic mobility shift studies of *Cpr* promoter fragments with tobacco nuclear extract. **A** Schematic representation of the promoter fragments used in EMSA experiments. The fragments (A to H, with their co-ordinates indicated) were obtained by using restriction sites or were derived from Erase-a-base deletion constructs previously generated for sequence analysis of the *Cpr* promoter. A map of the *Cpr* promoter from −1516 to +50 is shown at the top. The TATA box, putatitive ATG start codon (at position +1) and transcription start site (arrow) are indicated. The co-ordinates refer to the 5′ deletion end points in the series of *Cpr-gusA* fusion constructs that were analyzed in vivo (Fig. 4). **B** In vitro binding of nuclear factor GT-1 to fragment E. ^{32}P-labelled fragment E was incubated with 0.5 μg of tobacco nuclear extract. Competitor DNAs were added in molar excess as indicated. **C** Fragment E competes for GT-1 binding to box 4II. Probe 4II was incubated with 0.25 μg of tobacco nuclear extract. Fragment E competitor was added in 200-fold molar excess and 4II and 4IIm1 competitors in 100-fold molar excess. **D** GT-1 binding to promoter fragments A to H. Fragment E probe was incubated with 0.5 μg of tobacco nuclear extract. Fragments A to H were used as competitors in 100-fold molar excess

shown in Fig. 5B, the fragment E shift was effectively competed by the addition of unlabelled fragment E or box 4II, whereas the 4IIm1 and 4IIm2 mutants did not compete. In the reverse experiment, fragment E was shown to be capable of competing for the formation of a GT-1 complex with the 4II probe (Fig. 5C). In conclusion, mobility as well as specificity indicate that the complex observed with fragment E is due to binding of nuclear factor GT-1. To investigate whether GT-1 also binds to other regions in the *Cpr* promoter, fragments A to H were tested in a competition experiment with fragment E as a probe. As shown in Fig. 5D, the far upstream promoter fragments A, B and C strongly competed for GT-1 binding to fragment E. Fragment D, which is located directly upstream of fragment E, competed somewhat less efficiently, but fragment F, which is adjacent to the 3′ end of E, again showed strong competition. Finally, fragments G and H, which cover *Cpr* mRNA leader sequences, did not compete for GT-1 binding. In summary, the results indicate that GT-1 binds to multiple sites in the *Cpr* promoter, but not to the downstream part of the leader sequence.

In *C. roseus*, elicitor induction of *Cpr* transcription is coordinately regulated with induction of the *Tdc* and *Str1* genes, which also operate in TIA biosynthesis, suggesting that the *Cpr*, *Tdc* and *Str1* promoters may interact with the same nuclear factors (Pasquali et al. 1992; Meijer et al. 1993a). In agreement with this possibility, EMSAs with *Tdc* and *Str1* promoter fragments also revealed the presence of multiple binding sites for GT-1 (Pasquali 1994; Ouwerkerk 1997).

The observation that GT-1 interacts in vitro with a *Cpr* promoter region with strong enhancing properties in vivo, is suggestive of a functional role for this factor in determining *Cpr* promoter strength. Since deletion of the upstream part of the *Cpr* promoter (−1510 to −632) had little or no effect on the overall level of gene expression, it is unclear whether this region actually contributes to *Cpr* promoter activity. The fact that GT-1 also interacts with this region in vitro, may indicate that regulatory sequences are redundant in the *Cpr* promoter. Functional redundancy is a common property of promoter sequences and is also observed in, among others, the *C. roseus Tdc* and *Str1* promoters (Pasquali 1994; Ouwerkerk 1997) and in the pea *Rbcs-3A* promoter (Kuhlemeier et al. 1987). In vitro binding of GT-1 to elements with functional roles has previously been found for genes such as the pea *RbcS-3A* gene (Green et al. 1988; Kuhlemeier et al. 1988; Lam and Chua 1990), and a number of defence-related genes, including the bean *Chs15* gene (Lawton et al. 1991), the tobacco *PR-1* gene (Buchel et al. 1996) and the *C. roseus Tdc* (Ouwerkerk 1997) and *Str1* (Pasquali 1994) genes. A role for GT-1 in photoregulation has also been proposed (Kuhlemeier et al. 1988; Lam and Chua 1990). However, the accumulating observations that associate GT-1 with defence genes (Lawton et al. 1991; Pasquali 1994; Buchel et al. 1996; Ouwerkerk 1997), suggest that GT-1 may also be involved in regulation of defence responses in different plants. To clarify the role of GT-1 in *Cpr* promoter regulation, precise mapping of the binding sites will be required, which will enable studies of the effects of mutations on gene expression.

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