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CPRF4a, a novel plant bZIP protein of the CPRF family: comparative analyses of light-dependent expression, post-transcriptional regulation, nuclear import and heterodimerisation

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Abstract Several DNA-binding proteins with conserved basic region/leucine zipper domains (bZIP) have been isolated from parsley. They all recognise defined *A*CGT-*c*ontaining *e*lements (ACEs), including $ACE^{PcCHSII}$ in the *L*ight *R*egulatory *U*nit LRU1 of the *CHS* promoter which confers light responsiveness. A new member of this Common Plant Regulatory Factor (CPRF) family, designated CPRF4a, has been cloned, which displays sequence similarity to HBP-1a from wheat, as well as to other plant bZIP proteins. CPRF4a specifically binds as a homodimer to $ACE^{PcCHSII}$ and forms heterodimers with CPRF1 but not with CPRF2. In adult parsley plants, *CPRF2* and *CPRF4a* mRNAs are found in all tissues and organs in which the chalcone synthase gene *CHS* is expressed. In protoplasts from suspension cultured cells, UV irradiation (290–350 nm) did not cause an increase in levels of *CPRF1*, *CPRF2*, or *CPRF4a* mRNA, whereas the corresponding CPRF proteins accumulated within 15 min of light treatment. Furthermore, the rapid light-mediated increase of CPRF proteins was insensitive to transcriptional inhibitors, suggesting that a post-transcriptional mechanism controls CPRF accumulation. CPRFs as well as *Arabidopsis thaliana* G-box binding factors (GBFs) are selectively transported from the cytosol into the nucleus, as shown in an in vitro nuclear transport system prepared from evacuolated parsley protoplasts, indicating that cytosolic compounds are involved in regulated nuclear targeting of plant bZIP factors.

Key words Chalcone synthase · bZIP transcription factors · Post-transcriptional regulation · In vitro nuclear transport system · UV light induction

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Introduction

Plants often respond to changes in environmental conditions by changing patterns of gene expression. Cultured parsley (*Petroselinum crispum*) cells have been used extensively to study alterations in gene expression after UV irradiation and treatments with fungal elicitors (Hahlbrock and Scheel 1989). UV light and elicitors activate the expression of a defined set of genes involved in the synthesis of UV-protective flavonoid glycosides (Ebel and Hahlbrock 1977) and furanocumarins, respectively, and both stimuli are able to induce genes of the general phenylpropanoid pathway (Hahlbrock and Scheel 1989). The first committed step in flavonoid synthesis is catalysed by chalcone synthase (CHS). The transcriptional activation of this single-copy gene (Herrmann et al. 1988) is stimulated strongly by UV light in the range between 290 and 350 nm (UV-B: 280–320 nm; UV-A: 320–380 nm) and this response can be modulated by blue light (Ohl et al. 1989; Frohnmeyer et al. 1994). The dissection of the *CHS* promoter by DNA footprinting and transient expression analysis in protoplasts has revealed four *cis*-acting elements which are required for UV light-dependent *CHS* expression (Schulze-Lefert et al. 1989; Merkle et al. 1994). Two promoter regions, each containing two co-operating *cis*-elements were shown to act as light regulatory units (LRUs). LRU1, consisting of MRE^{PcCHS} (Box I; Feldbrügge et al. 1997) and the ACGT-containing element $ACE^{PcCHSII}$, is necessary and sufficient to confer light responsiveness on a heterologous core promoter (Block et al. 1990; Weisshaar et al. 1991; for nomenclature see also Feldbrügge et al. 1994). Several proteins that bind

to ACE^{PcCHSII} have been identified by South-western screening. They were designated *C*ommon *P*lant *R*egulatory *P*roteins (CPRFs) and belong to the family of bZIP proteins (Weisshaar et al. 1991). These proteins are characterised by a basic region-leucine zipper (bZIP) domain, which functions in dimerisation via the leucine zipper and in DNA contact via the basic region (Landschulz et al. 1988). DNA-binding by bZIP proteins always occurs with the dimeric form (Ellenberger 1994). So far, more than 30 plant bZIP proteins have been identified, all of which recognise ACEs. The specificity of DNA recognition by bZIP proteins is established by nucleotide triplets located 5′ and 3′ to the common ACGT core (Foster et al. 1994). While the majority of bZIP proteins are constitutively expressed in distinct plant tissues (Schindler et al. 1992a; Feldbrügge et al. 1996), some are regulated by external stimuli. For example, *CPRF1* mRNA expression is increased in parsley cell cultures and *GBF3* expression is decreased in *A. thaliana* upon irradiation with UV or white light (Weisshaar et al. 1991; Schindler et al. 1992b; Feldbrügge et al. 1994). *ZmGBF1* mRNA from maize (*Zea mays*) is induced by anaerobiosis (de Vetten and Ferl 1995), *OSZB8* mRNA from rice (*Oryza sativa*) is rapidly stimulated by abscisic acid (Nakagawa et al. 1996), and salicylic acid treatment of a tobacco (*Nicotiana tabacum*) cell culture results in the formation of a protein complex at the *as-1* site of the cauliflower mosaic virus *35S* promoter (Jupin and Chua 1996). However, a regulatory function for bZIP proteins at target promoters *in planta* has been described only for TGA1 from tobacco, CPRF1 from parsley and Opaque 2 from maize (Katagiri and Chua 1992; Lohmer et al. 1993; Feldbrügge et al. 1994).

As has been shown for TGA1, Opaque 2, and GBF1 from *A. thaliana*, the highly conserved basic domain of plant bZIP proteins not only confers DNA binding ability but also contains a bipartite *n*uclear *l*ocalisation *s*equence (NLS; van der Krol and Chua 1991; Varagona et al. 1992) which is responsible for nuclear transport of proteins synthesised in the cytoplasm through the nuclear envelope (reviewed in Davis 1995; Hicks and Raikhel 1995; Hicks et al. 1996; Terzaghi et al. 1997). Intracellular ACE-binding activities have been localised not only in the nucleus but also in the cytosol of parsley protoplasts, and light-dependent translocation of these cytosolic bZIP proteins has been demonstrated in an in vitro nuclear transport system (Harter et al. 1994). Similarly, blue-light induced import of *A. thaliana* GBF2 into nuclei of a soybean cell culture has been reported (Terzaghi et al. 1997).

In the present study, we describe the isolation and characterisation of CPRF4a from parsley, a new member of the plant bZIP family. We show that CPRF4a binds ACE^{PcCHSII} and forms heterodimers with CPRF1 but not with CPRF2 in vitro. The expression of CPRFs was monitored in plant tissue, cell cultures and protoplasts at the mRNA and protein levels, and compared with *CHS* gene activity. Finally, we show that CPRF1, CPRF2, and GBF2, but not CPRF4a or GBF1, is transported from the cytosol into the nucleus under these conditions, raising the possibility that additional post-transcriptional regulatory mechanisms function in UV-mediated signal transduction.

Materials and methods

Standard molecular biological techniques

Nucleotide sequences were determined as previously described (Kawalleck et al. 1993) using premixed reagents (Pharmacia, Heidelberg). All new DNA sequences were verified by sequencing both strands. Single-stranded oligonucleotides were desalted after deprotection, and used directly for PCR and DNA sequencing. Complementary oligonucleotides designed for South-western screening experiments, cloning, and mobility-shift assays (EMSAs) were purified by PAGE and annealed to yield double-stranded DNA with ligation-compatible ends. Annealing and relative DNA concentrations were verified by PAGE. Standard buffers and solutions were prepared according to Sambrook et al. (1989).

Light sources

Suspension cultures, protoplasts and cell-free extracts were irradiated with UV light from one Osram W/73 bulb and two Phillips TL/18 bulbs with a fluence rate between 300 and 500 nm of 15.0 μmol/m^2 per s; blue light was obtained from the same bulbs using a cut-off filter at 400 nm (14.2 μmol/m^2 per s). The degree of transmission of the petri dishes was 50% at 300 nm. In protoplasts, *CHS* expression is significantly induced only by wavelengths between 290 and 360 nm. The spectra of all light sources have been described recently (Frohnmeyer et al. 1994). All experiments were carried out under dim green safe light generated as described by Schäfer (1977).

Plant material and irradiation conditions

Parsley (*Petroselinum crispum*) plants were grown for 3 weeks (seedlings) or 6 months (adult plants) in a greenhouse. Samples were taken about 4 h after onset of illumination. Plant material was frozen in liquid nitrogen immediately after harvesting and stored at −80° C before use. The cell suspension culture was grown in HA medium, subcultured weekly and protoplasts were prepared 5 days after subculturing (Dangl et al. 1987). For irradiation experiments aliquots of 6–8 × 10^6 cells were transferred to petri dishes and filled to a volume of 3 ml with HA medium containing 0.4 M sucrose. After irradiation, cell or protoplast suspensions were diluted twofold in 0.24 M $CaCl_2$ and collected by centrifugation for 30 s at 10000 × *g*. After removal of the supernatant the pellets were frozen in liquid nitrogen and stored at −80° C.

RNA isolation and Northern analysis

Total RNA from parsley plants was isolated as described previously (Feldbrügge et al. 1994). Frozen aliquots of 5–7 × 10^6 cells or protoplasts were thawed in 0.6 ml of RNA extraction buffer [50 mM TRIS-HCl pH 8.0, 0.3 M NaCl, 5 mM EDTA, 0.1% (v/v) aurintricarboxylic acid, 2% (v/v) SDS] and homogenised with a metal pestle for 30 s using a mechanical drive (Potter S; B.Braun, Melsungen). The cell extract was vigorously shaken for 5 min after addition of phenol/chloroform at room temperature. The aqueous phase was extracted twice with phenol/chloroform and the RNA precipitated with 5 M LiCl. The RNA was dissolved in distilled water and the concentration determined spectrophotometrically.

Gene-specific cDNA probes were labelled by random priming (Feinberg and Vogelstein 1983) using premixed reagents (Boehringer, Mannheim). RNA was fractionated in agarose-formaldehyde gels (Davis et al. 1986) and blotted onto Hybond N (Amersham-Buchler) or UV Duralon membranes (Stratagene, Heidelberg). Hybond N membranes were used for the detection of organ-specific *CPRF4a* mRNA, and hybridised for 16 h in 10% dextran sulfate, 1 M NaCl, 50 mM TRIS-HCl pH 7.5, 10× Denhardt's solution and 1% SDS at 65° C. UV Duralon membranes were used for all other mRNA analyses and hybridised for 12 h in 50 mM sodium phosphate pH 6.5, 10× Denhardt's solution, 5 × SSC, 0.2% SDS 100 µg/ml salmon sperm DNA and 50% formamide at 42° C. Both filter types were incubated with 10^7 cpm of labelled probe and washed in 0.2 × SSC/0.5% SDS at 60° C. X-ray films were exposed at −80° C with intensifying screens. Before rehybridisation, membranes were stripped by incubation in 0.1% SDS solution for 20 min at 80° C. Hybridisation patterns were quantified with a Fuji bio-imaging analyser BAS1000 using PCBAS software (Raytest, Straubenhardt).

South-western screening of expression libraries

A new cDNA expression library was constructed in λZAPII as described (Weisshaar et al. 1991). cDNA was prepared using template RNA extracted with the Pharmacia Quick mRNA purification kit from 200 primary leaf buds of 3-week-old parsley seedlings which had been irradiated for 4 h prior to harvest with UV-containing white light. Approximately 3×10^5 plaque-forming units of the amplified library were screened as described (Somssich and Weisshaar 1996). The probe was a mixture of four different concatamerised, double-stranded oligonucleotides representing $ACE^{PcCHSII}$, $dACE^{PcCPRF1}$ (identical to ACE^{PcF1}), *as-1* and $dACE^{PcUBI4}$. The cDNA-containing pSK plasmid derivative of the λZAPII phage was recovered as the phagemid intermediate, and the insert was subjected to sequence analysis.

Screening of cDNA libraries by hybridisation

To obtain the full-length ORF of CPRF4a and sequence information on the 5′ untranslated region, a λZAPII library containing directionally cloned parsley cDNAs (Weisshaar et al. 1991) was screened using the original CFRF4a cDNA as a probe. Positive candidates from the first round of hybridisation were analysed by PCR to determine the size of a 5′ terminal fragment extending into the vector, using the T3 primer and an internal primer. Four candidates containing additional 5′ sequences relative to the South-western isolate were plaque purified, converted into plasmid form, and the cDNA inserts sequenced. One of the four cDNAs recovered was identical to the original isolate in the overlapping region and contained sequences from the 5′ part of the transcript; the three other cDNAs encoded a protein that is closely related to CPRF4a, which was designated CPRF4b.

Construction of expression clones and analysis by coupled in vitro transcription/translation

To generate the various expression plasmids, appropriate restriction sites were engineered into the CPRF4a cDNA at the start and at the end of the ORF (*Nco*I at the ATG; *Bgl*II immediately upstream of the stop codon) by PCR. To create F4t, a *Nco*I site was inserted in the CPRF4a ORF at codons 304 and 305. The F4t polypeptide contains amino acids 306–407 of CPRF4a. The PCR fragments were cloned using the pCR-Script cloning kit (Stratagene) and sequenced completely to exclude PCR-derived errors. The various ORFs were inserted into pBSexp (da Costa e Silva et al. 1993) for expression by coupled in vitro transcription/translation. In vitro transcription and subsequent in vitro translation reactions were carried out as described (Weisshaar et al. 1991).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed essentially as described (Armstrong et al. 1992). The oligonucleotide probes are identical to those used by Armstrong et al. (1992) and Feldbrügge et al. (1994).

Antisera and native bZIP protein extracts

The ORFs of GBF2 (Schindler et al. 1992a), CPRF1, CPRF2, (Weisshaar et al. 1991) and CPRF4a containing the appropriate restriction sites (5′ end: *Bam*HI; 3′ end: *Sal*I) were produced by PCR using specific primers and the ORF-containing plasmids as templates. Primer sequences are available on request. In the case of CPRF2, a full-length cDNA was used that had been isolated in the same way as described above for the full-length CPRF4a cDNA. The complete CPRF2 ORF has 402 amino acids, and the complete sequence is available from GenBank under accession number X58577. The PCR products were purified, cleaved with *Bam*HI/*Sal*I, and ligated into the vector pQE30 (Qiagen, Hilden), yielding constructs that express full-length CPRF1, CPRF2, CPRF4a and GBF2 proteins with an N-terminal $(His)_6$ tag. Proteins were expressed in *E. coli* strain M15 [pREP] by induction with 1 mM IPTG. Native protein extracts were obtained by resuspension of *E. coli* cells in 3 ml/mg of extraction buffer (60 mM HEPES/KOH pH 8.0, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 5 mM benzamidine, 1 mM ε-aminocapronate, 2 mM PMSF, 1 µg/ml antipain, 1 µg/ml leupeptin) and disruption in a French press at 4° C and 1000–1500 bar. Crude extracts containing high concentrations of CPRFs or GBFs were clarified by centrifugation at 4° C for 20 min at 100 000 × g.

For the production of antisera, the recombinant proteins were purified on Ni-NTA resin (Qiagen, Hilden) in the presence of 6 M guanidine hydrochloride and 8 M urea, according to the manufacturer's protocol. Proteins eluted from the Ni-NTA resin were separated by preparative SDS-PAGE. The gels were incubated in ice-cold 0.1 M KCl for 30 min to visualise proteins. The protein of interest was excised, electroeluted and stored at −80° C. 100 µg of purified protein was used for immunisation of mice. Three booster injections were subsequently administered. The specificity of all antisera was tested by immunoblotting using the various recombinant bZIP proteins as targets.

Protein extraction and immunodetection

Aliquots of frozen protoplast pellets ($1–2 \times 10^6$) were vigorously shaken and extracted with 100 µl of SDS sample buffer [65 mM TRIS-HCl pH 7.8, 4 M urea, 10 mM DTE, 5.0% (w/v) SDS, 0.05% (w/v) bromphenol blue] at 95 °C for 10 min and these crude extracts were clarified by centrifugation at 20 000×*g* for 15 min at 25° C. Total protein amounts, fractionation of protein extracts by SDS-PAGE, protein blotting and immunodetection were carried out as described by Frohnmeyer et al. (1992).

In vitro GBF/CPRF translocation assay and preparation of nuclei

Evacuolation of protoplasts was performed as described by Frohnmeyer et al. (1994). Cells were counted in a Fuchs-Rosenthal haemacytometer. For in vitro translocation assays dark-incubated, evacuolated protoplasts were permeabilised in lysis solution (16.8 µl/10^6 cells) containing 0.24 M sucrose 0.02% Triton X-100, 2 mM PMSF, 1 µg/ml antipain and 1 µg/ml leupeptin, and supplemented with a ATP-generating system (50 mM creatine phosphate, 2 U/µl creatine phosphate kinase). Native extracts containing recombinant bZIP proteins were added to 3×10^6 permeabilised cells (2 µg GBF1; 2 µg GBF2; 1 µg CPRF1; 2 µg CPRF2; 2 µg CPRF4a). Permeabilised cells ($2.5–3.5\times10^6$ cells/probe) were subsequently irradiated with UV light for 40 min at

25 °C. Nuclei were isolated as follows: lysates were mixed with 0.5 ml of ice-cold Ficoll buffer [20 mM MES/KOH pH 5.8, 20% (w/v) Ficoll, 20 mM potassium acetate, 0.5 mM spermidine, 0.15 mM spermine, 0.6 μM antipain, 0.6 μM leupeptin), diluted with 4 ml of ice-cold wash buffer (20 mM MES/KOH pH 5.8, 0.25 M sucrose, 20 mM potassium acetate, 2.0% (w/v) Dextran 40, 0.5 mM spermidine, 0.15 mM spermine, 1 μg/ml antipain, 1 μg/ml leupeptin, 2 mM PMSF, 0.05% Triton X-100) and centrifuged at 4° C for 5 min at 2000 rpm. The nuclei-containing pellet was washed twice in a total volume of 1.3 ml and 1 ml, respectively. The pellet was then resuspended in 100 μl of wash buffer, layered on 1 ml of Ficoll buffer and centrifuged for 5 min at 2000 × *g* (4° C). Sedimented nuclei were resuspended in 150 μl of proteinase K solution (0.1 mg/ml), incubated for 15 min at room temperature, and washed with 1 ml of wash buffer. Finally, the nuclei were extracted at 95° C with SDS sample buffer and nuclear proteins were analysed by immunoblotting using the various anti-GBF/CPRF antisera.

Results

Isolation of cDNA encoding CPRF4a

To obtain cDNAs encoding additional proteins which specifically bind ACE^{PcCHSII}, we employed the Southwestern technique to screen a λZAPII cDNA expression library constructed with RNA extracted from primary leaf buds of parsley seedlings irradiated for 4 h with UV-containing white light. Two independent phage plaques were isolated using concatamerised double-stranded ACE^{PcCHSII} as labelled ligand. Both phages contained cDNA fragments encoding proteins that recognise the wild-type ACE^{PcCHSII}. Sequence-specific DNA recognition was verified by using a probe derived from the oligonucleotide mACE^{PcCHSII} (for sequences see Feldbrügge et al. 1994). This mutant probe was bound by previously isolated non-specific DNA-binding proteins expressed from control phages (Weisshaar et al. 1991), but was not recognised by proteins expressed from phages encoding CPRF4a (data not shown). One of the cDNAs turned out to correspond to *CPRF1*, while the other coded for a new protein designated CPRF4a. Since the *CPRF4a* cDNA was incomplete, it was used to screen for longer cDNAs by DNA hybridisation. In addition to the complete *CPRF4a* cDNA (Genbank Accession No. Y10809), this screen yielded an additional cDNA which codes for a closely related protein designated CPRF4b (Accession No. Y10810). CPRF4b contains a stretch of 14 alanine residues in the N-terminal region and is otherwise 86 % identical to CPRF4a. Only the CPRF4a sequence was used for further investigations (Fig. 1).

CPRF4a specifically binds to the ACGT-containing element from the parsley *CHS* promoter (ACE^{PcCHSII})

To investigate the DNA-binding characteristics of CPRF4a, the protein was expressed by coupled in vitro transcription/translation. Electrophoretic mobility shift assays (EMSAs) revealed binding to ACE^{PcCHSII} (Fig. 2), thereby confirming the data from the Southwestern screening procedure. The specificity of the binding reaction was demonstrated in competition experiments using the unlabelled wild-type (Fig. 2, lanes 4–6; successful competition) or mutated ACE^{PcCHSII}

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1   MGSSEMEKSS KETKEPKTPT SQEQVSPVVA GPAGPVTPDW
                    motif I
41  SGFQAYSPMP PHGYMASSPQ APHPYMWGVQ HMMPPYGTPP
                    motif I
81  HPYVMYPHGG IYAHPSMPPG SYPFSPFAMP SPNGVAAEAS
                    motif VII
121 GNTPGSMEAD GGKVSEGKEK LPIKRSKGSL GSLNMITGKT
                                motif V
161 NEASKPSGAA TNGGYSKSGE SASEGSSEEG SDANSQNDSQ
201 IKSGSRQDSL EAGASHNGNA HGLQNGQYAN NSMVNQPISV
                        motif IV
241 VPLSTAGPTA VLPGPATNLN IGMDYWGGAT SSAIPAMRGQ
281 VSPPITGGTV SAGARDNVQS QLWLQDEREL KRQKRKQSNR
321 ESARRSRLRK QAECDELAQR AEALKEENAS LRAELSRFRT
361 EYEKIVAQNE VLKEKIREVP GQEDQWPGRN DQHNGNGSRE
401 TGHTEPA*
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Fig. 1 Deduced amino acid sequence of the novel parsley bZIP protein CPRF4a. The prolines of the N-terminal proline-rich region are indicated in *bold*, the basic domain in *bold, italic capitals*, and the leucines of the adjacent leucine zipper are in *bold* and *underlined.* Amino acid motifs listed by Meier and Gruissem (1994) are highlighted in *dashed boxes*

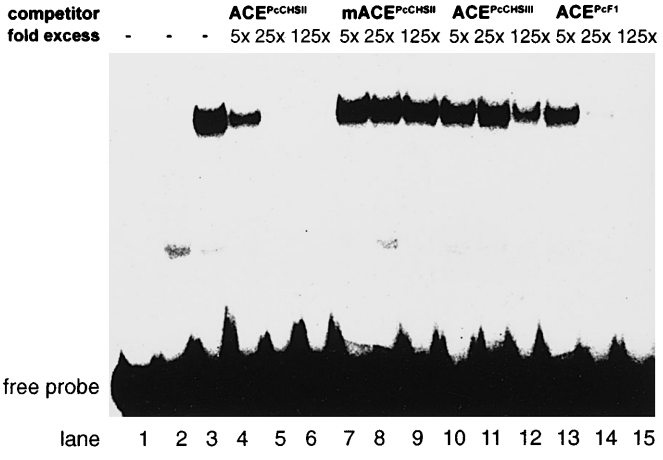

Fig. 2 Differential binding of CPRF4a to various parsley ACEs. Autoradiograph of an EMSA using radiolabelled ACE^{PcCHSII} oligonucleotides incubated with either no protein (lane 1), reticulocyte lysate without added mRNA (lane 2), or reticulocyte lysate programmed with *CPRF4a* mRNA (lanes 3–15). Unlabelled competitor DNA was added in the indicated excess over probe. mACE^{PcCHSII} is a mutated version of ACE^{PcCHSII} in which the ACGT core has been altered (lanes 7–9); ACE^{PcCHSIII} is the ACE motif from LRU2 of the parsley *CHS* promoter (lanes 10–12); ACE^{PcF1} is dACE^{PcCPRF1} from the 5′-region of the CPRF1 gene (lanes 13–15). In addition to the CPRF4a-specific complex, an unspecific band of higher mobility is detected when reticulocyte lysate is present in the binding reaction (compare lane 2)

(mACE^{PcCHSII}, lanes 7–9; no competition) sequences. Additional competition experiments demonstrated that CPRF4a bound to ACE^{PcFI} (lanes 13–15), an ACE present in the 5′ region of the *CPRF1* gene, but not to ACE^{PcCHSIII} (formerly Box III, lanes 10–12 in Fig. 2). Taken together, these results demonstrate that CPRF4a homodimers specifically recognise ACE^{PcCHSII} in vitro, and displays a preference for G-box-like ACEs.

CPRF4a selectively forms heterodimers

The dimerisation of CPRF4a was also analysed by EMSA (Fig. 3). Co-translation of mRNA encoding full-length CPRF4a (F4) with a truncated variant (encoding F4t, see Materials and methods) resulted in the formation of an additional complex which migrated with intermediate mobility when compared to F4 and F4t (Fig. 3, lanes 3–5), indicating the formation of an F4/F4t dimer. Co-translation of F4t with full-length CPRF1 (F1) also resulted in an additional complex with ACE^{PcCHSII} (lanes 6–8). In contrast, no additional complexes were observed after co-translation of CPRF2 (F2) and F4t (lanes 9–11), although dimerisation of these proteins without DNA binding may still occur. From these data we conclude that CPRF4a in vitro binds ACEs as a homodimer, and that CPRF4a selectively forms heterodimers with CPRF1 but not with CPRF2 at ACEs.

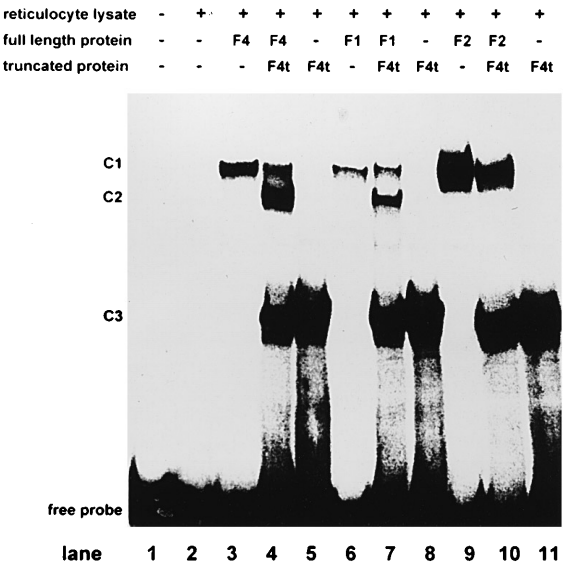

Fig. 3 CPRF4a selectively forms heterodimeric DNA-binding complexes. Designations and controls (lane 1 and 2) are similar to those used in Fig. 2. The reticulocyte lysate used in the binding reactions was programmed with in vitro transcribed mRNA encoding full-length CPRF4a (F4), CPRF1 (F1), CPRF2 (F2), truncated CPRF4a (F4t), or combinations of these mRNAs as indicated above the autoradiograph. *Arrowheads* indicate CPRF/ACE complexes: CPRF4a homodimers (C1), F4/F4t dimers, F1/F4t-heterodimers (C2), and F4t homodimers (C3)

Distribution of *CPRF4a* and *CPRF2* mRNA in organs of parsley seedlings and adult plants

The spatial pattern of expression of *CPRF4a* and *CPRF2* mRNA in parsley plants was determined by Northern analysis (Fig. 4). Under the conditions used, cross-hybridisation between the different *CPRFs* was not detected. The results obtained with the control probes for *CHS* and *UBI4* were identical to those obtained during the analysis of *CPRF1* expression (Feldbrügge et al. 1996). In seedlings, similar levels of *CPRF4a* and *CPRF2* mRNAs were detected in roots, cotyledons and primary leaves. A stronger or slightly increased signal, respectively, was observed for *CPRF4a* and *CPRF2* in primary roots. Lower levels of *CPRF4a* and *CPRF2* mRNAs were detected in primary leaves of seedlings, in which *CHS* shows a high expression level (Feldbrügge et al. 1996). In adult plants, *CPRF4a* and *CPRF2* mRNA was detectable in all organs. Except for relatively reduced *CPRF2* expression in the pedicel and in old leaf petioles, the distribution pattern of these factors was similar. We conclude from these data that *CPRF4a* and *CPRF2* mRNAs are present in all organs in which *CHS* mRNA is detectable, although there is no direct spatial correlation between the activities of these three genes.

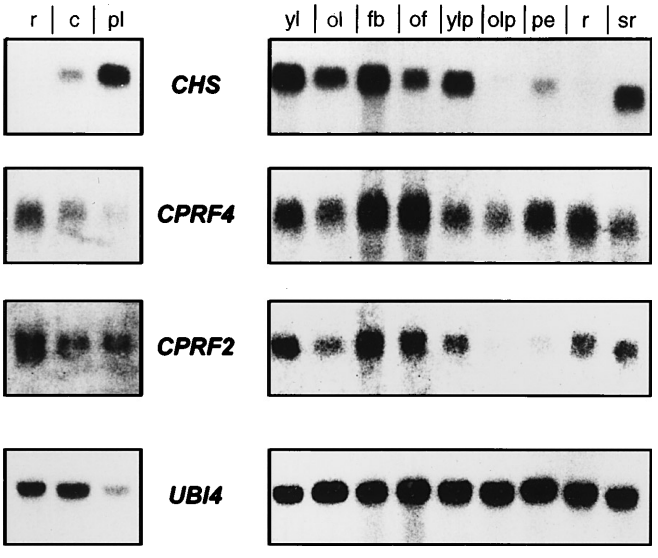

Fig. 4 Organ-specific accumulation of *CPRF2* and *CPRF4a* mRNAs in parsley seedlings and adult plants. Autoradiographs of Northern analyses of RNA isolated from organs of seedlings (*left panels*) and adult plants (*right panels*) are shown. Total RNA (10 μg) from root (r), cotyledons (c) and primary leaves (pl) of seedlings, and young leaves (yl), old leaves (ol), flower buds (fb), open flowers (of), young leaf petioles (ylp), old leaf pedicel (olp), pedicel (pe), roots (r) and storage roots (sr) was fractionated electrophoretically and transferred to Hybond N nylon membrane. The membrane was first hybridised with the *CPRF4a* probe (designated as *CPRF4*) and subsequently rehybridised with *CPRF2*, *CHS*, and *UBI4* probes

CPRF mRNA abundance in parsley protoplasts is independent of light treatment

Recent studies with cultured parsley cells have revealed light-dependent accumulation of *CPRF1* and constitutive expression of *CPRF2* mRNA (Weisshaar et al. 1991). An important advantage of this cell culture system is the maintenance of light responsiveness in isolated protoplasts, which allows one to study light responses using transient transformation with chimeric *CHS* promoter/reporter gene constructs (Dangl et al. 1987; Schulze-Lefert et al. 1989). It was therefore of interest to compare *CPRF* expression patterns in this system under various light regimes. Cultured cells or freshly prepared protoplasts were kept in the dark or irradiated with UV and blue light (BL). After 15 min, 60 min and 6 h, total RNA was isolated and subjected to Northern analysis using *CPRF4a* and *CHS* probes. Irrespective of the light regime, cells and protoplasts express high levels of *CPRF4a* mRNA. In contrast, *CHS* expression is strongly induced after 6 h of treatment with UV light and only weakly induced by BL (Fig. 5).

Levels of *CPRF1*, *CPRF2*, and *CPRF4a* mRNAs after irradiation with UV light were determined in freshly prepared protoplasts. Expression levels were measured at three time points (15 min, 60 min and 6 h) in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX, 50 μM). CHX is known to inhibit UV-stimulated *CHS* mRNA accumulation and to enhance the *CPRF1* mRNA level in cultured parsley cells (Feldbrügge et al. 1996). The accumulation of *CPRF1*, *CPRF2* and *CPRF4a* mRNAs was investigated by Northern analysis, and results from two or three independent experiments were quantified by phosphoimaging (Fig. 6). Irradiation of protoplasts with UV light does not significantly alter *CPRF1* mRNA levels up to 6 h, while *CHS* mRNA accumulated strongly, as expected (see Fig. 5). Blue light, which causes only a weak induction of *CHS* mRNA in protoplasts (Fig. 5), led to a slight reduction in *CPRF1* mRNA levels (data not shown). In contrast, dark-incubated protoplasts treated with CHX showed a dramatic increase in *CPRF1* mRNA within 15 min and levels had increased by five-fold after 6 h, while UV irradiation of CHX-treated protoplasts caused only a two-fold increase in *CPRF1* mRNA levels (Fig. 6). *CPRF2* mRNA accumulated similarly under these conditions. UV light (as well as blue light, not shown) failed to cause any increase in *CPRF2* mRNA after 15 min, 60 min or 6 h. Incubation with CHX led to a strong increase in *CPRF2* mRNA in

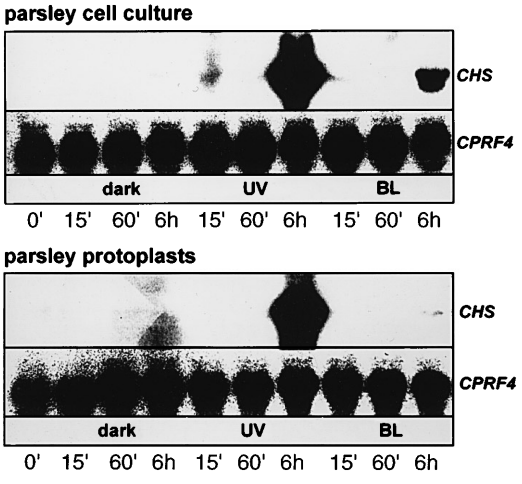

Fig. 5 Accumulation of *CPRF4a* mRNA is not light regulated in cultured parsley cells and protoplasts. Autoradiographs of Northern analyses of RNA isolated from cultured parsley cells (*upper panel*) and protoplasts (*lower panel*) are shown. Cells were irradiated with UV and blue light (BL) for 15 min, 60 min and 6 h, or incubated in the dark. Aliquots (20 μg) of total RNA were fractionated electrophoretically and transferred to Duralon membrane. The membrane was first hybridised with the *CPRF4a* probe (*CPRF4*) and subsequently reprobed with the *CHS* probe

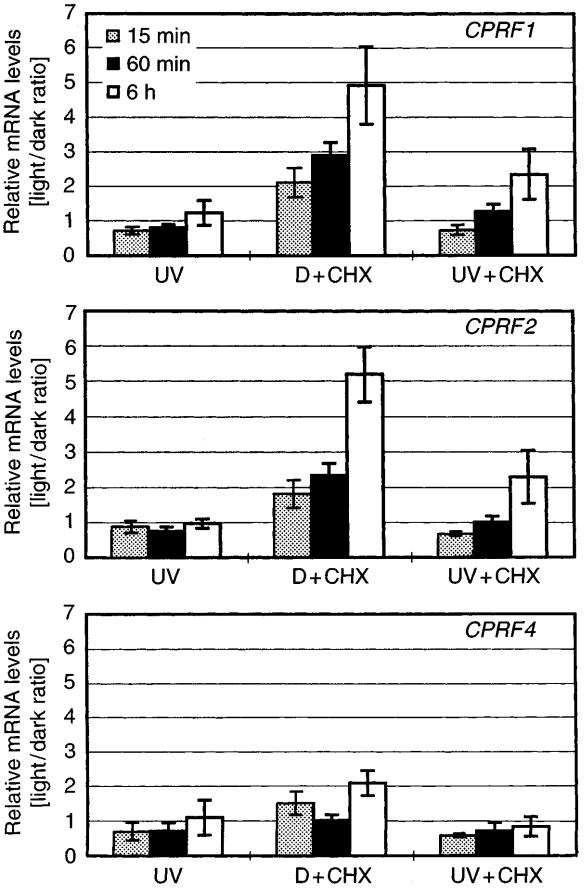

Fig. 6 Relative *CPRF* mRNA accumulation in parsley protoplasts. Quantified data of two independent Northern analyses of RNA isolated from parsley protoplasts are shown. Cells were irradiated with UV light (UV) for 15 min, 60 min and 6 h, or incubated in the dark (D). In parallel, aliquots were pre-treated with 50 μM cycloheximide for 30 min prior to UV irradiation (UV + CHX) or incubation in the dark (dark + CHX). Aliquots (20 μg) of total RNA were fractionated electrophoretically, transferred to Duralon membranes and subsequently hybridised with *CPRF1*, *CPRF2* and *CPRF4a* (*CPRF4*) probes. Results were quantified by phosphoimaging and the corresponding values for incubation in the dark defined as 1. Error bars indicate the standard errors of the means

the dark, while no increase was detectable during UV irradiation (Fig. 6). *CPRF4a* mRNA showed a comparable expression pattern in UV-irradiated protoplasts, while the addition of CHX led to only a two-fold accumulation after 6 h (Fig. 6).

CPRF1, CPRF2, and CPRF4a proteins accumulate transiently in parsley protoplasts during irradiation with UV or blue light

To analyse CPRF protein accumulation by immunoblot analysis, specific polyclonal antisera were produced in mice against His-tagged recombinant parsley CPRF1, CPRF2 and CPRF4a proteins. The detection limit of the antisera was found to be 5 ng for CPRF1, 10 ng for CPRF2 and below 2.5 ng for CPRF4a (data not shown). Parsley protoplasts were treated as described for the analysis of mRNA levels, except that cordycepin, which is known to block the elongation step during transcription (Romero and Lam 1993), was used as an inhibitor instead of CHX. Crude protein extracts were fractionated by SDS-PAGE and the amounts of CPRFs, CHS, and of an unknown parsley bZIP protein detectable with the anti-GBF1 antiserum (Harter et al. 1994) were determined.

As shown in Fig. 7, CPRF1 and CPRF2 were found to be expressed at low levels in dark-incubated protoplasts (lanes 1–4). Surprisingly, UV irradiation of protoplasts caused a strong increase in CPRF2 and CPRF4a protein levels within 15 min (lane 5). The highest amounts of all CPRF proteins were found after 60 min, while after 6 h of UV irradiation only CPRF1 was detectable and CHS had accumulated strongly (lanes 6–7). Pre-incubation with cordycepin (100 μM) blocked the UV light-dependent increase in CHS levels completely, while expression of CPRF and GBF was not inhibited but rather continued up to 6 h after the start of the UV treatment (lanes 8–10). Blue light led to an increase in CPRF protein levels within 60 min, as found for UV treatment, but did not cause accumulation of CHS protein (lanes 11–13). Expression of the GBF1-related protein from parsley was constitutive under all conditions (Fig. 7). Coomassie blue staining of the gels confirmed equal loading of all lanes (data not shown). Taken together, these data suggest that UV light and blue light cause a rapid increase in levels of CPRF1, CPRF2 and CPRF4a proteins, which is independent of transcription.

CPRF1, CPRF2, and GBF2, but not CPRF4a or GBF1, are translocated into the nucleus in vitro

Cytosolic fractions of cultured parsley cells contain a strong ACE binding activity, suggesting a retardation of nuclear factors potentially involved in the light signal transduction in the cytosol (Harter et al. 1994). It was therefore of interest to determine whether different bZIP proteins participate in this mechanism and whether a selective transport mechanism functions between the cytosol and the nucleus. To analyse this mechanism, we modified the in vitro nuclear transport system (Harter et al. 1994) and monitored the cytosolic retention (or nuclear import) of recombinant parsley CPRFs and *A. thaliana* GBF1 and GBF2. Our in vitro transport system is based on evacuolated protoplasts (EP), which still activate *CHS* gene expression after UV irradiation. The plasmalemma of EP was permeabilised with 0.02% Triton-X–100, a concentration which does not affect organelle membranes, and the system was supplied with an energy-regenerating system (Frohnmeyer et al. 1994). After addition of recombinant bZIP protein (CPRF1, CPRF2, CPRF4a, GBF1 or GBF2), the lysate was irradiated for 45 min with UV light. Nuclei were immediately extracted from the lysate, and remaining extranuclear proteins were degraded with proteinase K. This procedure ensured that only recombinant proteins which have been transported into the nucleus were detected. Immunoblot analysis of such extracts revealed selective transport of the various bZIP proteins. Full-length CPRF1 and CPRF2 proteins, as well as two smaller peptides that cross-react with the respective antibodies, were found to be imported into the nucleus (Fig. 8). In contrast, full-length CPRF4a remained in

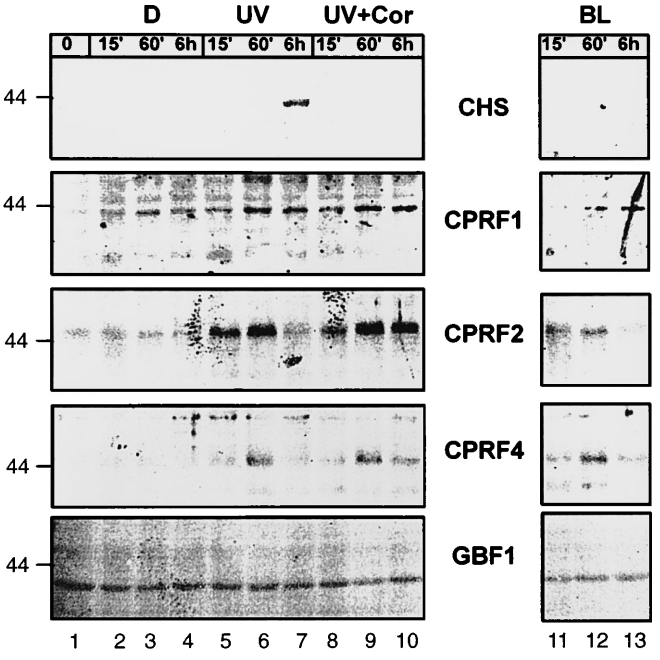

Fig. 7 Light-dependent, transient accumulation of CPRF1, CPRF2 and CPRF4a protein in parsley protoplasts. Results of immunoblot analyses using protein extracts isolated from parsley protoplasts are shown. Protoplasts were irradiated with UV light (UV) or blue light (BL) for 15 min, 60 min and 6 h, or incubated in darkness (D). UV + Cor indicates that cells were incubated with 150 μM cordycepin for 30 min prior to UV irradiation. After harvesting, total protein was extracted and samples (20 μg/lane) were fractionated by SDS-PAGE. Proteins were detected with specific antisera against parsley CHS, CPRF1, CPRF2 and CPRF4a (CPRF4) and *A. thaliana* GBF1. The position of the molecular mass standard is indicated in kDa

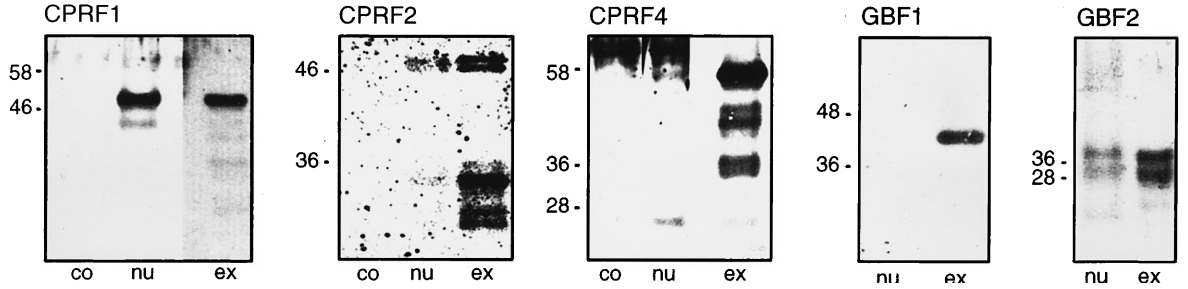

Fig. 8 CPRF1, CPRF2 and GBF2, but not CPRF4a or GBF1, are translocated into parsley nuclei. Western blot analysis of in vitro nuclear transport assays are shown. Preparation of the lysates and the isolation of nuclear fractions are described in the text. Nuclear extracts with (nu) or without (co) recombinant protein and, as a control, the respective overexpressed bZIP alone (ex) were separated by SDS-PAGE and the amounts of bZIP protein in the nuclear fraction was analysed by immunoblotting. Proteins were detected with specific antisera against parsley CHS, CPRF1, CPRF2 and CPRF4a (CPRF4) as well as with *A. thaliana* GBF1 and GBF2. The positions of molecular mass standards are indicated in kDa

the cytosol, while a 25-kDa CPRF4a fragment was detectable in the nuclear fraction. Recombinant heterologous GBF1, supplied to the in vitro system was retained in the cytosol, while GBF2 was transported into the nucleus (Fig. 8). These data indicate that plant bZIP proteins, which have been shown to contain a bipartite NLS in the basic region, are selectively retained in the cytosol.

Discussion

Structure of CPRF4a

By sequence comparison, CPRF4a can be classified as a typical member of the bZIP family of DNA-binding proteins. Its amino acid sequence contains two motifs known from other ACE binding proteins: the characteristic bZIP domain consisting of a basic region (amino acids 311–330) located next to a leucine zipper (amino acids 337–372), and a proline-rich region located in the N-terminal portion of the protein that is not involved in DNA-binding or dimerisation. Sequence alignments with other plant bZIP proteins showed 50% sequence identity of CPRF4a with wheat (*Triticum aestivum*) HBP-1a (Tabata et al. 1991), 42% with GBF1 and 40% with maize OBF1 (Foley et al. 1993). However, less sequence identity was found between CPRF4a and CPRF1 (34%) or CPRF2 (16%). The stronger resemblance of CPRF4a to bZIPs from other species, compared to parsley CPRF1 and CPRF2, suggests that these proteins have different physiological functions. A similar conclusion can be drawn from the plant bZIP factor classification scheme of Meier and Gruissem (1994). Based on the presence of distinct sequence motives, CPRF4a belongs to their B class, while CPRF1 is a member of the C class and CPRF2 represents an independent class.

CPRF4a binds ACE^{PcCHSII} as a homodimer, and as a heterodimer with CPRF1 but not with CPRF2

All bZIP transcription factors investigated so far form homo- or heterodimers during DNA binding. In agreement with this, CPRF4a can form homodimers and heterodimers with CPRF1. In contrast, no interaction with CPRF2 was observed. CPRF1 shows a comparable heterodimerisation profile (Armstrong et al. 1992). These data suggest a complex binding pattern at ACE^{PcCHSII}: CPRF1 and CPRF4a can bind as homo- and heterodimers, whereas CPRF2 only binds as a homodimer. These results, and the low level of sequence similarity of CPRF2 to CPRF4a and CPRF1, further indicate that CPRF2 is a distinct member of the bZIP factor family.

In parsley, overexpression of CPRF1 represses the light-induced *CHS* promoter activity, an effect which may be caused by squelching (Feldbrügge et al. 1994). Many parallel studies in animal systems have reported that heterodimer formation leads to specific changes in gene expression. One example is provided by the bZIP proteins JUN and FOS, which bind to AP-1 sites present in many promoters. Both the JUN homodimer and the JUN/FOS heterodimer bind to AP-1 sites, but the transcriptional activation potential of the JUN/FOS heterodimer is stronger (reviewed in Karin and Hunter 1995).

Tissue-specific and light-dependent CPRF expression

CPRF1 mRNA is detectable in all organs of parsley seedlings and adult plants in which *CHS* mRNA is present (Feldbrügge et al. 1996), and a similar pattern was observed in this study for *CPRF2* and *CPRF4a*. However, *CPRF* mRNAs were also detected in roots and old leaf petioles, where *CHS* mRNA is only poorly expressed. No direct spatial correlation can be observed between *CHS* expression and the presence of a distinct ACE^{PcCHSII}-binding CPRF, pointing to a broader functional relevance of CPRFs, which may not be restricted to light regulation of *CHS* gene expression (Logemann et al. 1995; see also below).

Parsley protoplasts, which maintain their light responsiveness after digestion of the cell walls express similar levels of *CHS* mRNA as cultured cells (Dangl et al. 1987). It was therefore of interest to study the endogenous *CPRF* expression under different light regimes. Considerable amounts of *CPRF1*, *2*, and *4* mRNAs were already detectable in dark-incubated protoplasts, and blue or UV light treatment caused no significant changes in expression. While *CPRF2* and *CPRF4a* are expressed similarly in the cultured cells and in isogenic seedlings, *CPRF1* mRNA expression is light induced in these systems (Weisshaar et al. 1991; Feldbrügge et al. 1994). A direct comparison of *CPRF1* mRNA accumulation in cultured cells and protoplasts showed clearly reduced induction of mRNA levels in protoplasts (data not shown). One reason for this difference could be that protoplast formation, i.e. digestion of the cell wall, may lead to increased *CPRF1* gene expression. Independently of these transcriptional events, the accumulation of CPRF proteins is rapidly induced by light in protoplasts, indicating a post-transcriptional mechanism of control.

The role of CPRFs in UV light-mediated signal transduction

The rapid, light-dependent expression of CPRF proteins is in agreement with earlier experiments, in which a strong increase of $ACE^{PcCHSII}$-binding activity was found in UV-irradiated cytosol of parsley protoplasts (Harter et al. 1994). Binding of CPRFs to ACEs is regulated by phosphorylation (data not shown), as described for GBF1 (Klimczak et al. 1992), and kinase inhibitors block binding of CPRFs to $ACE^{PcCHSII}$, as well as UV light-induced *CHS* expression (Harter et al. 1994; Frohnmeyer et al. 1997). CPRF proteins accumulate in the cytosol within 15 to 30 min after UV irradiation (this work and Harter et al. 1994) and must be imported into the nucleus. Surprisingly, intracellular localisation seems to be regulated also, since CPRF1 and 2 are selectively transported into the nucleus after UV irradiation. CPRFs may be retained in protein complexes in the cytosol until a light stimulus selectively releases specific factors which are then imported into the nucleus. As a result, the effective nuclear concentration of each CPRF could be regulated in a light-dependent way, which could lead to formation of distinct homo- and heterodimers at the respective target promoters. Our in vitro data are in accordance with in vivo studies in soybean cells, which describe selective BL-dependent import of *A. thaliana* GBF2 into the nucleus (Terzaghi et al. 1997).

UV light-induced *CHS* gene activity in the parsley cell culture and in protoplasts was previously shown to be inhibited by the protein synthesis inhibitor cycloheximide (Hahlbrock and Ragg 1975; Feldbrügge et al. 1996; H. Frohnmeyer, unpublished observation), a finding which was also made in *A. thaliana* (Christie and Jenkins 1996). This result suggests that some components of signal transduction to the *CHS* promoter may be synthesised early after the onset of irradiation. *CPRF* mRNA accumulation is induced by CHX treatment in the dark, an observation which has also been reported for other transcription factors involved in hormone-, red light-, and salicylic acid-induced signalling (Lam et al. 1989; Ballas et al. 1993; Qin et al. 1994; Jupin and Chua 1996). The rapid, transient expression of CPRFs, in combination with the CHX-inducible accumulation of their respective mRNAs, makes these transcription factors good candidates for early genes involved in UV light-mediated signalling. However, the exact role of CPRFs in UV light-mediated *CHS* expression is still unclear: the expression of *CPRF* mRNA after imposition of different light regimes does not correlate with *CHS* expression in protoplasts, and the spatial distribution of these factors and the target gene is not congruent in all plant tissues. One possibility is that *CPRF*s are early genes, but are not exclusively or even predominantly responsible for *CHS* activation. CPRF1, for example, has been shown to bind to the ACE from the H3-7 gene promoter, which is negatively regulated by light (Feldbrügge et al. 1994; Logemann et al. 1995). In addition, the requirement for a second *cis*-acting element (MRE^{PcCHS}) next to the $ACE^{PcCHSII}$ for LRU1 function has to be considered (Schulze-Lefert et al. 1989; Feldbrügge et al. 1997). Similar interactions between *cis*-acting elements seem to be necessary for the activation of various light-, stress- and hormone-responsive promoters. The tobacco *RBCS* promoter, for example, needs a GGTTAA or GATAAG element as a partner, for its ACE to confer light responsiveness (Donald and Cashmore 1990), and ACEs of ABA-responsive promoters also need additional *cis*-elements to mediate stimulus-dependent promoter activity (de Vetten and Ferl 1994; Ono et al. 1996).

All these data imply that CPRFs act as transcriptional activators or repressors depending on the promoter context. The nuclear CPRF concentration is possibly regulated by nuclear import mechanisms (and/or retention in the cytosol). Their transcriptional activation potential may, furthermore, be modulated by the formation of homo- or heterodimers. Differential binding in various promoter contexts and CPRF interaction with other transcription factors could determine specific effects in plant signal transduction. Our results shed some light on the complex network of events in UV light-mediated signalling cascades, and they clearly show the involvement of post-transcriptional regulation at the level of translation and protein localisation. However, further studies are necessary to unravel the precise function of these factors.

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